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(54) Title: ESTROGEN SIGNALLING PATHWAY REGULATORS AND USES THEREOF

(57) Abstract

The present invention relates to estrogen receptor signalling and the regulation of estrogen receptor signalling for the treatment of cancer and proliferative disease. The invention further relates to the treatment of cancers associated with mutations in BRCA1 gene. Methods of identifying "estrogen receptor signalling pathway regulators" are also part of the invention. Therapeutic compositions and methods of screening, diagnosis and therapy are also part of the invention. Methods of the invention can be used to inhibit cancer cell growth, especially in cancers of the breast, ovaries and prostate.

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ESTROGEN SIGNALLING PATHWAY REGULATORS AND USES THEREOF

This application claims priority under 35 U.S.C. §119(e) to U.S. Application No. 60/131,841, filed April 30, 1999, the contents of which are incorporated herein by reference in its entirety.

1. FIELD OF THE INVENTION

The present invention relates to estrogen receptor signalling and regulation of estrogen receptor signalling for the treatment of cancer and proliferative disease. The invention relates to molecules and/or compounds that regulate the estrogen signalling pathway. More specifically, the invention relates to the treatment of cancers and diseases associated with alterations in hormone or hormone responsiveness. In a preferred embodiment, the invention relates to the treatment of breast cancer or cancers associated with mutations in the BRCA1 gene. The present invention relates, in part, to the discovery that BRCA1 inhibits signalling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element (ERE) and blocks the transcriptional activation function of ER-α. The invention relates to compositions and methods for treating cancer and proliferative disease, such as breast cancer. The invention further relates to regulation of the estrogen receptor signalling pathway with an "estrogen receptor signalling pathway regulator", or "ESP-regulator".

2. BACKGROUND OF THE INVENTION

2.1 BRCA1

BRCA1 was the first breast cancer susceptibility gene to be identified and cloned. In individuals from high-risk families, mutations in BRCA1 increase the lifetime risk of developing breast cancer eight to tenfold, compared to the general population. How the BRCA1 protein product normally functions to suppress tumor formation and how defects in the gene can ultimately lead to breast cancer have been the focus of intense scrutiny by the scientific and medical communities. BRCA1 has intrinsic transactivation activity and is able to activate the p21 promoter. In addition, BRCA1 is linked to a number of genes involved in transcriptional regulation, including CtIP, c-Myc, the RNA holoenzyme complex, and the histone deacetylase complex. Moreover, BRCA1 is essential for cellular response to DNA damage repair. Inactivation of BRCA1 in mouse embryonic stem and fibroblast cells results in increased cell sensitivity to DNA-damaging agents.

Germ-line mutations of the BRCA1 gene located on chromosome17q21 (Miki et al., 1994, *Science* 266:66) account for 40-50% of hereditary breast cancers and confer increased risk for ovarian and prostatic cancers (Ford et al, 1994, *Lancet* 343:692; Gayther et al., 1995, *Nat. Genet.* 11: 428; Streuwing et al., 1997, *New Engl. J. Med.* 336: 1401). The BRCA1 gene encodes an 1863 amino acid (aa) protein with a highly conserved N-terminal RING finger domain and a C-terminal acidic transcriptional activation domain (Miki et al.,1994, *Science* 266:66; Monteiro et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93: 13595; Chapman and Verma, 1996, *Nature* 382:678). The BRCA1 gene product is a 220 kD nuclear phosphoprotein (Chen et al., 1986, *Cancer Res.* 56:3168) that has been implicated in regulation of cell proliferation (Hakem et al., 1996, *Cell* 85:1009; Holt et al., 1996, *Nat. Genet.* 12:298), cell cycle progression (Chen et al., 1996, *Cancer Res.* 56:3168; Somasundaram et al., 1997, *Nature* 389:187; Larson et al., 1997, *Cancer Res.* 57:3351), apoptosis induction (Shao et al., 1996, *Oncogene* 13:1; Fan et al., 1998, *Oncogene* 16:3069) and DNA repair and recombination (Fan et al., 1998, *Oncogene* 16:3069; Scully et al., 1997, *Cell* 90:426; Husain et al., 1998, *Cancer Res.* 58:1120; Gowen, 1998, *Science* 281:1009). These functions of BRCA1 are observed in various human epithelial cancer cell types and mouse fibroblasts and do not explain the association of BRCA1 mutations with specific tumor types, such as breast, ovarian and prostate cancer.

2.2 ESTROGEN RECEPTOR

Nuclear hormone receptors are a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements. The two closely related human estrogen receptors (ER-α and ER-β), which belong to this family, were cloned and sequenced from MCF-7 human breast cancer cells (Green et al., 1986, *Nature* 320:134-139; and reviewed in Perlmann and Evans, 1997, *Cell* 90:391). The ER-α protein, the major estrogen receptor of mammary epithelia, consists of 595 amino acids with a molecular weight of 66 kD (Green et al., 1986) that has been separated into six different functional domains (Kumar et al., 1987, *Cell* 51:941-951; Kumar et al., 1986, *EMBO J.* 5:9931-9936). Two of these functional domains are highly conserved in the primary sequence of members of the nuclear hormone receptor superfamily. One of the domains, the DNA binding domain, contains two zinc fingers that mediate receptor binding to hormone response elements in the promoters of hormone-responsive genes. In the C-terminal region, the hormone binding domain contains two regions of sequence homology with other hormone receptors and bestows hormone specificity and selectivity

(Carson-Jurica et al., 1990, *Endocr. Rev.* 11:201-220; Kumar and Chambon, 1988, *Cell* 55:145-156; Orti et al., 1992, *Endocr. Rev.* 13:105-128). Estrogen responses are mediated by two closely related receptors, ER-α and ER-β (reviewed in Perlmann and Evans, 1997, *Cell* 90:391).

Estrogen diffuses through the plasma membrane of cells where it binds to the ER (Rao et al., 1981, *Mol. Cell. Endocrinol.* 21:97-108). For many years, it generally was thought that estrogen bound to the ER in the cytoplasm and translocated into the nucleus, but it is known now that the ER is a nuclear transcription factor that initially interacts with estrogen in the nucleus (Welshons et al., 1984, *Nature* 307:747-749). Once estrogen binds to the ER, heat shock proteins dissociate and a change in conformation and homodimerization occurs.

Once the ER has bound estrogen and dimerized, it binds to estrogen response enhancers (EREs) present in the promoter region of genes. These EREs are 13 base pair palindromic sequences located upstream from the transcriptional start site. The EREs function by enhancing the transcriptional potential of a gene. EREs have been identified and defined using reporter systems to test the enhancer ability when exposed to different compounds (Gronemeyer, 1991, *Annu. Rev. Genet.* 25: 89-123). Also, deletional analysis has allowed the definition of the sequence of EREs. The exact sequence of EREs varies between species and genes (Klein-Hitpass et al., 1986, *Cell* 46:1053-1061).

ER-dependent transcription can be enhanced by coactivators. The first candidate for a transcriptional coactivator, SPT6, was isolated from Saccharomyces cerevisiae and was shown to be capable of modulating ER-mediated transcription in yeast and mammalian cells and to interact specifically with the carboxyl-terminal portion of the ER (Baniahmad et al., 1995, *Mol. Endocrinol.* 9:34-43). A member of the estrogen receptor coactivating complex is p300. This co-factor, acting in concert with ligand binding, is responsible for the conformational change required of the ER-α activation. The amino-and carboxy-terminal transactivation domains of the ER (AF-1 and AF-2) can now interact and cause further recruitment of coactivator proteins. These coactivators link ER-α to the basal transcriptional machinery and convert chromatin to the active state via histone acetylation.

Another co-activator is the CREB-binding protein (CBP). This protein can interact specifically with members of the steroid hormone nuclear receptor family and is able to enhance transcriptional activity (Kamei et al., 1996, *Cell* 85:403-414). CBP has been shown to also interact with RNA polymerase II (Kee et al., 1996, *J. Biol. Chem.* 271: 2373-2375), TFIIB (Kwok et al., 1994, *Nature* 370: 223-226), and with cAMP response

element-binding protein (CREB) in its phosphorylated form (Chrivia et al., 1993, *Nature* 365:855-859). It has been postulated that the ability of CBP to stimulate transcription is through the targeted recruitment of RNA polymerase II to the promoters of genes.

3. SUMMARY OF THE INVENTION

The present invention relates to estrogen receptor signalling and the inhibition or regulation of estrogen receptor signalling for the treatment of cancer and proliferative disease. More specifically, the invention relates to the treatment of breast cancer or diseases associated with alterations in hormone or hormone responsiveness. The present invention relates, in part, to the surprising discovery that BRCA1 inhibits signalling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element (ERE) and blocks the transcriptional activation function of ER-α. The invention relates to compositions and methods for treating cancer and proliferative disease, such as breast cancer. The invention further relates to regulation of the estrogen signalling pathway as a method of treating and/or preventing breast cancer.

Mutations of the breast cancer susceptibility gene BRCA1 are linked to development of breast, ovarian, and prostatic cancers; but it is not clear why BRCA1 mutations lead to these particular tumor types. The present invention relates to the discovery that BRCA1 modulates signalling by the ligand-activated estrogen receptor (ER-α). Inhibition of ER-α activity is demonstrated herein to require at least three structural domains of BRCA1 including the N-terminal RING finger, a consensus retinoblastoma (RB) binding motif (^{358}LXCXE), and a third domain at the C-terminus. The discoveries of the present invention suggest that the transcriptional adaptor p300, which is a component of the ER-α coactivator complex, may be a target for repression of ER-α by BRCA1. The present invention relates to the discovery that the loss of the ability to regulate ER-α contributes to the incidence of breast cancer in BRCA1 mutation carriers. Thus, the methods and compositions of the present invention are useful for the treatment or amelioration of breast cancer or precancerous conditions by inhibition or regulation of the estrogen pathway in hosts in need of such treatment. In one embodiment, a host in need of treatment or amelioration of breast, ovarian or prostate cancer or precancerous condition carries a BRCA1 mutation. In other embodiments, a host is screened for a BRCA1 mutation prior to treatment with an estrogen signalling pathway regulator (ESP-regulator) of the present invention.

The present invention further provides for regulating the cellular proliferation of a cancer cell, dysplastic cell, tumorigenic cell, malignant cell, or

precancerous cell. The present invention also encompasses the use of combinations of two or more estrogen signalling pathway regulators.

At the molecular genetic level the coding sequence for an ESP-regulator may be placed under the control of one or more of the following genetic elements: a naturally occurring strong, intermediate or weak constitutively expressed or regulated promoter from the targeted tissue, or an artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that delivers desired levels of the estrogen signalling pathway regulator expression. In a preferred embodiment of the invention, tissue-specific promoters can be used to direct expression of an ESP-regulator in a particular tissue. For example, the whey acidic protein promoter can be used to direct expression of an estrogen signalling pathway regulator in breast tissue.

This genetic information is delivered into the target cells by a either a biologic or abiologic delivery vehicle. In one embodiment of the present invention, a biologic viral vector is used to deliver an ESP-regulator. Alternatively, an abiologic delivery system (e.g., liposomes) can be used to package nucleic acid carrying the genetic elements necessary and sufficient for the proper expression of the ESP-regulator(s) into liposomes.

The present invention further encompasses the use of the ESP-regulators of the present invention for the treatment of disease, such as cancer or hyperproliferative disorders. The present invention further relates to a method of treating a host having a proliferative disease of a specific tissue by inhibiting cell proliferation in the tissue, comprising administering to the host a ESP-regulator wherein the tissue-specific promoter binding sequence is specific for the diseased tissue, whereby the ESP-regulator encoded by the nucleic acid is expressed, estrogen receptor signalling is regulated in the tissue, cell proliferation is inhibited, and the proliferative disease treated is provided. The present invention encompasses the ESP-regulators of the present invention in pharmaceutical formulations.

The present invention further encompasses the use of the ESP-regulators of the present invention for research and screening purposes. In one embodiment of the present invention, the ESP-regulators may be used to screen for gene products or molecules which effectively inhibit the proliferation of a cancer cell (such as a breast cancer cell with a BRCA1 mutation).

In yet another embodiment, the present invention relates to a vector encoding an ESP-regulator.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Fig . 1. Inhibition of ER-α activity by BRCA1 and mapping of involved domains. **(a)** BRCA1 strongly inhibits ER-α signalling in prostate (DU-145) and breast cancer (T47D) cells. Cells were assayed for stimulation of ERE-TK-Luc reporter activity by 178-estradiol (E2). E2 induced a (100-1000)-fold increase in reporter activity when expression vector pSG5-ER-α (ER-α) was provided. wtBRCA1 but not empty vector (pcDNA3), ablated E2-stimulated reporter activity. A control reporter missing the ERE (TK-Luc) gave no E2-stimulated activity. Note: Similar results were obtained whether assays were carried out in serum free-medium (as shown) or in DMEM supplemented with 2% charcoal-stripped serum (data not shown). **(b)** BRCA1 weakly inhibits ER-α signalling in human cervical cancer cell lines. In three cervical cancer cell lines (C33A, CaSki, and SiHa), E2-stimulated reporter activity in the presence of wtBRCA1 was reduced to 38-51% of that in the presence of empty vector. In contrast, two other prostate cancer cell lines (LNCaP and TsuPr-1) and two other breast cancer cell lines (MCF-7 and MDA-MB-231) showed virtual ablation of reporter activity by wtBRCA1. **(c)** Diagrams of proteins encoded by *BRCA1* genes. Functional domains shown are: BRCT= BRCA1 C-terminal homology region; RING= ring finger domain; LXCXE= consensus retinoblastoma binding motif; NLS1= nuclear localization signal; TAD= transactivation domain. **(d)** Expression of BRCA1 genes in DU-145 cells by semi-quantitative RT-PCR. All BRCA1 cDNAs are expressed well. In addition, the BRCA1 transgenes produce proteins of expected size when translated (data not shown). **(e)** Effect of BRCA1 genes on ER-α activity in DU-145 and T47D. Activity is expressed as % of empty pcDNA3 vector positive control. Values are means ± SEMs of at least three experiments. **(f)** Dose-response for inhibition of ER-α activity by wtBRCA1 vs 5677insA. The total transfected DNA content was kept constant by addition of the empty pcDNA3 vector. Values are means ± SEMs of quadruplicate wells. **(g)** BRCA1 inhibits the estrogen-stimulated expression of the endogenous pS2 and cathepsin D genes in MCF-7 (top panel) and T47D (bottom panel) breast cancer cells. Cells were transfected overnight with wtBRCA1 or empty pcDNA3 vector, washed, and incubated in serum-free DMEM (5.0 ml per 100 mm dish) +/-E2 (1 μM). Conditioned medium was concentrated 10-fold and 1/10 of the concentrate (≅50 μl) was used for Western blotting.

Fig. 2. *In vivo* association of BRCA1 and ER-α. **(a)** Association of endogenous BRCA1 and ER-α in untransfected cells. Nuclear lysates from proliferating cells were subjected to immunoprecipitation (IP)-Western blotting using antibodies against BRCA1, ER-α, or control (mouse IgG). ER-α was detected in the BRCA1 IP of MCF-7 cells and vice versa.

but not in the BRCA1 IP of DU-145 cells, which are ER negative. **(b)** Association of BRCA1 and ER-α in transiently transfected DU-145 cells. Transfections were carried out under conditions parallel to those of the transcription assays. When expressed in DU-145 cells, ER-α does associate with BRCA1. **(c)** Restoration of ER-α signaling by BRCA1ΔEcoRI. In inhibition of ER-α signaling by wtBRCA1 could be decreased with the addition of increasing amounts of BRCA1ΔEcoRI.

Fig. 3. *In vitro* binding of BRCA1 to ER-α by GST capture. **(a)** Schematic diagrams of ER-α proteins tested in GST binding assays (Wang et al., 1993, *Science* 260:1330-1335). **(b)** GST:ER fusion proteins visualized by Western blotting. **(c)** Binding of *in vitro* translated (IVT) wtBRCA1 to beads coated with GST-ER fusion proteins. The input lane shows 10% of the IVT wtBRCA1 used in each assay. **(d)** IVT products of different BRCA1 cDNAs (see Fig. 1c), demonstrated by autoradiography. **(e)** Binding of IVT BRCA1 proteins to beads coated with GST-ER 282-420. **(f)** Relative binding of different BRCA1 proteins to ER 282-420. Bars show the ratio of bound: input BRCA1 derived from densitometric analysis of the data in panels d and e.

Fig. 4. wtBRCA1 inhibits expression of p300 in prostate and breast cancer cells. **(a)** wtBRCA1 down-regulates p300 levels in DU-145 cells. Subconfluent proliferating cells in 100 mm dishes were incubated overnight with 15 μg of each vector in 5.0 ml of DMEM containing Lipofectamine. Cells were washed, incubated in fresh DMEM for 24 hr and harvested for Western blotting. Of the various BRCA1 genes tested, only wtBRCA1 and 5677insA inhibited p300 expression, while none of these genes altered CBP expression **(b)** wtBRCA1 and BRCA1-5677insA also inhibited p300 expression in T47D cells but was still incapable of affecting CBP levels. **(c)** Unlike DU-145 and T47D, three cervical cancer cell lines (C33A, CaSki and SiHa) did not decrease the levels of p300 (or CBP) in response to wtBRCA1. Note: SiHa cells had no detectable p300, but showed a band of slightly lower M_r, consistent with p27O, a p300-like protein that cross-reacts with the p300 antibody (Dallas et al., 1996, *J Virol* 71:1726-1731).

Fig. 5. p300 and CBP overrides *wtBRCA1* inhibition of ER-α activity. **(a)** Diagrams of proteins encoded by p300 genes. Domains shown are: CH1, CH2, CH3 = cysteine/histidine rich regions-1, -2, -3; HAT = histone acetyl transferase domain; PCAF = p300-interacting cofactor; SRC-1 = steroid receptor coactivator-1. **(b)** p300 overrides the inhibition of ER-α by wtBRCA1. Co-transfection of wildtype (wt-p300) or mutant p300 genes missing the

HAT or Bromo domains blocked the inhibition by wBRCA1. A p300 mutant missing the CH3 domain failed to block the inhibition; while a mutant consisting of only the CH3 region plus flanking sequences (p300 1514-1922) did override the inhibition. **(c)** Wild-type CBP (wt-CBP) also overrides wtBRCA1 inhibition of ER-α activity. **(d)** Effect of co-transfection of both wtBRCA1 and p300 on p300 levels in DU-145 cells. Co-transfection of wt-p300 blocked the down-regulation of p300 by wtBRCA1; but co-transfection of p300 1514-1922 did not block down-regulation of endogenous full-length p300 by wtBRCA1. Neither wt-p300 nor p300 1514-1922 inhibited BRCA1 expression.

Fig. 6. The CR3 domain of CBP/p300 interacts with ER-α. **(a)** The carboxyl-terminal region of human CBP shows estrogen-independent binding to the AF-2/ligand binding domain region of mouse ER-α, as demonstrated by GST capture assay. **(b)** The binding site for ER-α maps to the CH3 region of human CBP. On the other hand, GRIP1 binding maps to the p160-interacting site located carboxyl-terminal to CH3. **(c)** Human ER-α binds to the CH3 region of CBP independently of estrogen. Estrogen (17β-estradiol) and the estrogen antagonists (tamoxifen, raloxifene, and ICI 182,780) were used at a concentration of 0.1 μM. **(d)** A p300 fragment containing the CH3 domain (p300 1514-1922) binds to ER 282-420. Competition assays in which GST-ER 282-420 was preincubated with IVT p300 1514-1922 or IVT wtBRCA1 showed reduced binding of wtBRCA1 and p300 1514-1922, respectively. The amounts of competing protein represent μg of plasmid DNA. As a control, pre-incubation of GST-ER 282-420 with similar quantities of IVT empty vector (pcDNA3) reaction product failed to inhibit the binding of p300 1514-1922 to GST-ER 282-420.

Fig. 7. Model for breast cancer suppression of BRCA1. Our findings suggest a model in which the amino-terminus of BRCA1 interacts with ER-α, and the carboxyl-terminus acts as an transcription inhibition domain. BRCA1 and p300/CBP interact with the AF2/LBD region of ER-α. We propose that p300/CBP compete with BRCA1 for a binding site on ER-α or otherwise neutralizes the ability of BRCA1 to inhibit AF-2. The failure of mutant forms of BRCA1 to inhibit ER-α activity can contribute to inappropriate cellular proliferation and breast cancer.

5. DETAILED DESCRIPTION OF THE INVENTION

Mutations of the breast cancer susceptibility gene BRCA1 are linked to development of breast, ovarian, and prostatic cancers; but it is not clear why BRCA1

mutations lead to these particular tumor types. The present invention relates to the discovery that BRCA1 inhibits signalling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element (ERE) and blocks the transcriptional activation function of ER-α. Inhibition of ER-α activity requires at least three structural domains of BRCA1: the N-terminal RING finger, a consensus retinoblastoma (RB) binding motif (^{358}LXCXE), and a third domain at the C-terminus. The studies describe herein suggest that transcriptional adaptor p300, which is a component of the ER-α coactivator complex, is a target for repression of ER-α by BRCA1. The loss of the ability to regulate ER-α may contribute to the incidence of breast cancer in BRCA1 mutation carriers. Further, the present invention is based, in part, on the Applicants' discovery that the failure to repress ER-α activity may contribute to breast cancer.

The present invention relates to the estrogen signalling pathway and inhibition or regulation of estrogen receptor signalling as a means of treating the proliferative disease. More specifically, the invention relates to the treatment of breast cancer or cancers associated with alterations in hormone or hormone responsiveness. The present invention relates, in part, to the discovery that BRCA1 inhibits signalling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element (ERE) and blocks the transcriptional activation function of ER-α. The invention relates to compositions and methods that regulate estrogen receptor signalling for treating cancer and proliferative disease, such as breast cancer. In particular, the invention relates to compositions and methods that regulate ER-α activity as a treatment for cancer or proliferative disease. The compositions of the invention include, but are not limited to, estrogen signalling pathway regulator (herein called an "ESP-regulator"). The invention further relates to regulation of the estrogen signalling pathway. In accordance with the invention, methods and compositions of the invention are used for the treatment of a host having one or more BRCA1 mutations. The invention relates to the repression of ER-α activity as a treatment for cancer or proliferative disease.

An ESP-regulator of the invention may be any molecule (such as a compound, small molecule, nucleic acid, gene product, protein, hormone, recombinant protein, synthetic molecule, etc.) that is capable of modulating the estrogen signalling pathway. Such regulators include but are not limited to members of the estrogen signalling pathway (such as the ER-α, ER-β), ligands of an estrogen receptor (such as estrogen, recombinant estrogen, or modified forms of estrogen *e.g.*, modified to bind the receptor with decreased efficacy), molecules which bind the estrogen receptor and prevent translocation of the receptor or receptor/ligand complex into the nucleus, molecules which bind to the DNA-

binding domain of the receptor and inhibit signalling, molecules which prevent receptor dimerization, molecules which bind to the transactivation domain and inhibit transactivation, molecules which bind to the ligand binding domain and inhibit signalling. Such regulators also include dominant negative proteins (such as a dominant negative estrogen receptor). Such regulators may also include molecules which act to compensate for a BRCA1 mutation, or a molecule which interacts directly with a BRCA1 protein to enhance BRCA1 protein regulation of the estrogen receptor activation or signalling.

The present invention further relates to the use of a wide variety of vehicles to deliver the ESP-regulator of the invention to a target or target tissue, including virions and viral vectors to package and deliver the DNA encoding an ESP-regulator non-viral expression vectors and abiologic vehicles, including liposomes and liposome-DNA and lipid-DNA complexes to deliver and target the DNA encoding an ESP-regulator to the host.

In accordance with the invention, the host to which the ESP-regulator is delivered may be cells in culture, tissues in culture, animal models, animals, mammals, or preferably humans. In a preferred embodiment, the ESP-regulator is delivered to a female human host. In a more preferred embodiment, the ESP-regulator is delivered to a female human host, having breast cancer or at risk of acquiring breast cancer (such as one who has been identified as a carrier of a BRCA1 mutation).

The present invention relates to pharmaceutical compositions comprising the ESP-regulators of the present invention and their delivery vehicles. The ESP-regulators of the present invention may be engineered for the treatment of a wide variety of disorders and diseases related to expression of a particular gene or genes, cellular overproliferation, hereditary disorders, cancers, tumors, or proliferative disorders.

The present invention further relates to methods of screening hosts for BRCA1 mutations in order to identify those individuals that are more susceptible to developing cancer, in particular breast, ovarian or prostate cancer. The present invention also relates to methods of screening hosts to identify and diagnose individuals carrying BRCA1 mutations that are in a precancerous stage or having cancer, in particular breast, ovarian or prostate cancer. The present invention further relates to methods of screening hosts known to have cancer, in particular breast, ovarian or prostate cancer, to identify those individuals carrying BRCA1 mutations that would benefit from methods of treatment and compositions described herein. Individuals to particularly benefit from treatment are those containing mutations in BRCA1 domains essential for function (*e.g.*, the N-terminal RING finger domain, the consensus retinoblastoma binding motif and a domain in the C-terminal 550 amino acids).

In another embodiment the present invention also relates to *in vitro* screening or research purposes, *e.g.*, to identify gene products involved in cellular overproliferation such as a BRCA1 mutation, or an estrogen signalling pathway gene mutation. For example. *in vitro* screening may be used to identify mutations that compensate for a BRCA1 mutation and thus serve to protect from overproliferation due to the BRCA1 mutation.

5.1 PRECANCEROUS/PREMALIGNANT CONDITIONS

The invention provides for the screening of individuals to ascertain their BRCA1 nucleotide sequence and therefore the degree to which they are at risk of developing cancer, especially of the breast. ovary or prostate. Individuals found to be at risk can be treated prophylactically to decrease their chance of developing cancer. The invention also provides for the treatment of such precancerous or premalignant conditions. In one embodiment, precancerous or premalignant conditions is treated by induction of apoptosis. The precancerous or premalignant cells in which apoptosis is induced are generally ones which exhibit at least one functional p53 allele. "Functional" as used herein. refers to an ability of the p53 allele to contribute to differential apoptosis in cells. It is to be noted that in certain instances. administration of the tumor suppressor of the invention results in restoration of mutant p53 protein conformation and/or activity to normal. Thus. while precancerous or premalignant cells exhibiting at least one functional p53 allele are preferred targets of the methods of the invention, the methods described herein are not to be limited to such cells.

Precancerous or premalignant cells include. but are not limited to cells which present in conditions known or suspected to precede progression to neoplasia or cancer. in particular. where non-neoplastic cell growth consisting of hyperplasia. metaplasia. or most particularly. dysplasia has occurred (for review of such abnormal growth conditions. *see* Robbins and Angell. 1976. *Basic Pathology*. 2d Ed.. W.B. Saunders Co.. Philadelphia. pp. 68-79).

Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ. without significant alteration in structure or function. As but one example. endometrial hyperplasia often precedes endometrial cancer.

Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. As but one example. the esophageal metaplasia of Barrett's esophagus often precedes esophageal cancer.

Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the skin, cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of therapeutic administration of the methods and compositions of the invention.

As mentioned above, such characteristics of a transformed phenotype include morphology changes, loss of differentiation markers, reversion to a dedifferentiated phenotype, cell rounding, loss of adhesion, decreased spreading, loss of polarity, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, etc (*see* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 84-90 for characteristics associated with a transformed or malignant phenotype).

In accordance with the invention, an ESP-regulator modulates estrogen receptor signalling activity or expression is used to treat or prevent hyperproliferative or benign dysproliferative disorders. Specific embodiments are directed to treatment or prevention of benign tumors, fibrocystic conditions, and tissue hypertrophy (*e.g.*, prostatic hyperplasia).

5.2 GENE THERAPY

The present invention provides the therapeutic or prophylactic use of an ESP-regulator. In a specific embodiment, nucleic acids comprising a sequence encoding an ESP-regulator or functional derivative thereof, are administered to regulate or inhibit cell proliferation, by way of gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a host. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by to regulating or inhibiting cell proliferation of a cancerous, precancerous, of diseased cell.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, *see* Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, *TIBTECH* 11:155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; and Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY.

In a preferred aspect, the Therapeutic comprises an ESP-regulator nucleic acid that is part of an expression vector that expresses a an ESP-regulator protein or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the ESP-regulator coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the ESP-regulator coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the ESP-regulator nucleic acid (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (*see* U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand host to receptor-mediated endocytosis (*see e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand

complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

In a specific embodiment, a viral vector that contains the ESP-regulator nucleic acid is used. For example, a retroviral vector can be used (*see* Miller et al., 1993, *Meth. Enzymol.* 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The ESP-regulator nucleic acid to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302; Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114. Adenoviruses are other viral vectors that can be used in gene therapy. Targets for adenovirus-based delivery systems are epithelial cells, liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. (see, Kozarsky and Wilson, 1993, *Current Opinion Gen. Dev.* 3:499-503). Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300.

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such

that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In one embodiment, recombinant cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art. In accordance with the invention, delivery of such recombinant cells may be particularly desirable when used in combination with surgery or surgical treatment administered for the treatment of a tumor, a cancer, or a proliferative disease. For example, in a preferred embodiment of the invention, recombinant cells expressing an ESP-regulator are administered during or after a mastectomy.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc. In a preferred embodiment, the cell used for gene therapy is autologous to the patient. In an embodiment in which recombinant cells are used in gene therapy, a ESP-regulator nucleic acid is introduced into the cells such that they are expressed by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect.

5.3 ASSAYS OF ESP-REGULATORS

The functional activity of ESP-regulators, derivatives and analogs can be assayed by various methods known to one skilled in the art.

For example, in one embodiment, where one is assaying for the ability of an ESP-regulator or an estrogen-receptor antibody to bind or compete for binding to an estrogen receptor, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or

radioisotope labels), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.* In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. In another embodiment, where a estrogen receptor binding protein is identified, the binding can be assayed, *e.g.*, by means well-known in the art. In another embodiment, physiological correlates of ESP-regulator binding to its substrates (*e.g.*, signal transduction molecules) can be assayed.

In another embodiment, an ESP-regulator or estrogen receptor is assayed for the ability to self association as a dimer, tetramer, or multimer. Assays for self-association include but are not limited to separation of complexes on a sucrose gradient or centrifugation gradient, analysis on a non-denaturing electrophoretic gel, or *in vitro* gel mobility shift assays. In another embodiment, detection of the association and disassociation of protein complexes is measured by Fluorescence Polarization (In *Physical Biochemistry: Applications to Biochemistry and Molecular Biology* 2nd Ed., Freifelder, 1982, W.H. Freeman and Company, New York). Methods for detecting complexes may also be performed as described in Stenger et al., 1992, *Mol. Carcinog.* 5:102-106, including analysis by gradient gel electrophoresis and chemical cross-linking. In one embodiment, full cross-linking is combined with denaturing gel electrophoresis. In another embodiment. partial cross-linking is performed followed by dissociating tetramer complexes into monomers, dimers and trimers.

In yet another embodiment, the subcellular localization of an ESP-regulator or estrogen receptor may be examined by any assay known in the art. For example, immunofluorescence staining with antibodies against an ESP-regulator protein can reveal the subcellular location of such protein. In a further embodiment, cells may be simultaneously stained for known markers of subcellular compartments, such that the location of the ESP-regulator protein may be co-localized by fluorescence. In another embodiment, physiological correlates of the subcellular location of an ESP-regulator protein (*e.g.*, nuclear) can be assayed.

Another functional assay of a ESP-regulator is an assay for the ability of the ESP-regulator to bind DNA or nucleotide sequence, or the ability of an ESP-regulator to

inhibit the binding of an estrogen receptor to DNA. Such assays include the electrophoretic mobility shift assay.

Other methods will be readily apparent to the skilled artisan and are within the scope of the invention.

5.4 BIOCHEMICAL ASSAYS USING AN ESP-REGULATOR

The present invention provides for biochemical assays using the ESP-regulators of the invention. In one embodiment, ESP-regulators are useful for biochemical assays aimed at the identification and characterization of molecules which interact with a estrogen signalling pathway proteins, or are new estrogen signalling pathway proteins (e.g. proteins in the same signaling pathway as an estrogen receptor). For example, the cDNAs encoding the ESP-regulator proteins can be individually subcloned into any of a large variety of eukaryotic expression vectors permitting expression in insect and mammalian cells, described above. The resulting genetically engineered cell lines expressing ESP-regulator proteins can be assayed for production, activation, and interactions of ESP-regulator proteins, for example with antibodies ESP-regulator protein and Western blotting assays or ELISA assays. For assays of specific binding and functional activation of estrogen receptors or ESP-regulators, one can employ either crude extracts or lysates containing protein from genetically engineered cells, or partially purified extracts, or preferably highly purified ESP-regulator protein fractionated, for example, by chromatographic methods. Alternatively, an ESP-regulator protein can be synthesized using chemical methods (Nagata et al., 1992, *Peptides* 13:653-62).

Specific binding of an ESP-regulator to an estrogen receptor (*e.g.*, ER-α) can be assayed as follows, for example, following the procedures of Yamaguchi et al. (Yamaguchi et al., 1995, *Biochemistry* 34:4962-4968). Chinese hamster ovary cells, COS cells, or any other suitable cell line, can be transiently transfected or stably transformed with expression constructs that direct the production of an estrogen receptor. Direct binding of an ESP-regulator to such ER-α-expressing cells can be measured using a "labeled" purified ESP-regulator derivative, where the label is typically a chemical or protein moiety covalently attached to the ESP-regulator which permits the experimental monitoring and quantitation of the labeled ESP-regulator in a complex mixture.

Specifically, the label attached to the ESP-regulator can be a radioactive substituent such as an ^{125}I-moiety or ^{32}P-phosphate moiety, a fluorescent chemical moiety, or labels which allow for indirect methods of detection such as a biotin-moiety for binding by avidin or streptavidin, an epitope-tag such as a Myc-or FLAG-tag, or a protein fusion

domain which allows for direct or indirect enzymatic detection such as an alkaline phosphatase-fusion or Fc-fusion domain. Such labeled ESP-regulator can be used to test for direct and specific binding to ER-α-expressing cells by incubating the labeled ESP-regulator with the ER-α-expressing cell lysates or subcellular fractions. Also, instead of a direct binding assay, a competition binding assay may be used.

This invention provides assays for detecting changes in the expression of estrogen signalling pathway genes and proteins. Such estrogen signalling pathway genes and proteins include but are not limited to genes and proteins within the estrogen pathway as well as estrogen responsive gene (e.g., genes that are transcriptionally activated by the ligand-activated estrogen receptor). Assays for changes in gene expression are well known in the art (*see e.g.*, PCT Publication No. WO 96/34099, published October 31, 1996, which is incorporated by reference herein in its entirety). Such assays may be performed *in vitro* using transformed cell lines, immortalized cell lines, or recombinant cell lines, or *in vivo* using animal models.

In particular, the assays may detect the presence of increased or decreased expression of estrogen signalling pathway genes and proteins on the basis of increased or decreased mRNA expression (using, *e.g.*, nucleic acid probes), increased or decreased levels of related protein products (using, *e.g.*, the antibodies disclosed herein), or increased or decreased levels of expression of a marker gene (*e.g.*, β-galactosidase or luciferase) operably linked to a 5' regulatory region in a recombinant construct.

In yet another series of embodiments, various expression analysis techniques may be used to identify genes which are differentially expressed between two conditions, such as a cell line or animal expressing a normal BRCA1 gene compared to another cell line or animal expressing a mutant BRCA1 gene. Such techniques comprise any expression analysis technique known to one skilled in the art, including but not limited to differential display, serial analysis of gene expression (SAGE), nucleic acid array technology, subtractive hybridization, proteosome analysis and mass-spectrometry of two-dimensional protein gels.

To elaborate further, the various methods of gene expression profiling mentioned above can be used to identify other genes (or proteins) that may have a functional relation to (*e.g.*, may participate in a signaling pathway with) an estrogen signalling pathway gene. Gene identification of such other genes is made by detecting changes in their expression levels following mutation, *i.e.*, insertion, deletion or substitution in, or overexpression, underexpression, mis-expression or knock-out, of a BRCA1 gene, as

described herein. Expression profiling methods thus provide a powerful approach for analyzing the effects of mutation in a BRCA1 gene or ESP-regulator gene.

Methods of gene expression profiling are well-known in the art, such as exemplified by the following references describing differential display (Liang and Pardee, 1992, *Science* 257:967-971), proteosome analysis (Humphery-Smith et al., 1997, *Electrophoresis* 18:1217-1242; Dainese et al., 1997, *Electrophoresis* 18:432-442), SAGE (Velculescu et al., 1995, *Science* 270:484-487), subtractive hybridization (Wang and Brown, 1991, *Proc. Natl. Acad. Sci. U.S.A.* 88:11505-11509), and hybridization-based methods of using nucleic acid arrays (Heller et al., 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:2150-2155; Lashkari et al., 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:13057-13062; Wodicka et al., 1997, *Nature Biotechnol.* 15:1259-1267).

5.5 PROLIFERATION AND CELL CYCLE ASSAYS

An ESP-regulator has potential implications in regulation of cell proliferation and control of the cell cycle. Further, a number of signaling pathway molecules relating to ESP-regulator and cell cycle regulation are of interest in the instant invention. The present invention provides for cell cycle and cell proliferation analysis by a variety of techniques known in the art, including but not limited to the following techniques.

As one example, bromodeoxyuridine (BRDU) incorporation may be used as an assay to identify proliferating cells. The BRDU assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (*see* Hoshino et al., 1986, *Int. J. Cancer* 38:369; Campana et al., 1988, *J. Immunol. Meth.* 107:79).

Cell proliferation may also be examined using [^{3}H]-thymidine incorporation (*see e.g.*, Chen, 1996, *Oncogene* 13:1395-403; Jeoung, 1995, *J. Biol. Chem.* 270:18367-73). This assay allows for quantitative characterization of S-phase DNA synthesis. In this assay, cells synthesizing DNA will incorporate[^{3}H]-thymidine into newly synthesized DNA. Incorporation can then me measured by standard techniques in the art such as by counting of radioisotope in a Scintillation counter (*e.g.* Beckman LS 3800 Liquid Scintillation Counter).

Detection of proliferating cell nuclear antigen (PCNA) may also be used to assay cell proliferation. PCNA is a 36 kilodalton protein whose expression is elevated in proliferating cells, particularly in early G1 and S phases of the cell cycle and therefore may serve as a marker for proliferating cells. Positive cells are identified by immunostaining

using an anti-PCNA antibody (*see* Li et al., 1996, *Curr. Biol.* 6:189-199; Vassilev et al., 1995, *J. Cell Sci.* 108:1205-15).

Cell proliferation may be measured by the counting samples of a cell population over time (*e.g.* daily cell counts). Cells may be counted using a hemacytometer and light microscopy (e.g. HyLite hemacytometer, Hausser Scientific). Cell number may be plotted against time in order to obtain a growth curve for the population of interest. In a preferred embodiment, cells counted by this method are first mixed with the dye Trypan-blue (Sigma), such that living cells exclude the dye, and are counted as viable members of the population.

DNA content and/or mitotic index of the cells may be measured, for example, based on the DNA ploidy value of the cell. For example, cells in the G1 phase of the cell cycle generally contain a 2N DNA ploidy value. Cells in which DNA has been replicated but have not progressed thru mitosis (e.g. cells in S-phase) will exhibit polidy value higher than 2N and up to 4N DNA content. Ploidy value and cell cycle kinetics may further be measured using propidium iodide assay (*see e.g.* Turner, et al., 1998, *Prostate* 34:175-81). Alternatively, the DNA ploidy may be determined by quantitation of DNA Feulgen staining (which binds to DNA in a stoichiometric manner) on a computerized microdensitometrystaining system (*see e.g.*, Bacus, 1989, *Am. J. Pathol.*135:783-92). In an another embodiment, DNA content may be analyzed by preparation of a chromosomal spread (Zabalou, 1994, *Hereditas.*120:127-40; Pardue, 1994, *Meth. Cell Biol.* 44:333-351).

Further, the expression of cell cycle proteins (*e.g.*, CycA, CycB, CycE, CycD, CycJ, cdc2, Cdk4, Cdk6, E2f, E2f2, Rb, p21^{cip1}, p27, etc.) also provide crucial information relating to the proliferative state of a cell or population of cells. For example, identification in an anti-proliferation signaling pathway may be indicated by the induction of p21^{cip1}. Expression of p21 can be elevated by p53. In turn, increased levels of p21 expression in cells results in Cdk inhibition, resulting in delayed entry into G1 of the cell cycle (Harper et al., 1993, *Cell* 75:805-816; Li et al., 1996, *Curr. Biol.* 6:189-199). p21 induction may be identified by immunostaining using a specific anti-p21 antibody available commercially (*e.g.* Santa Cruz). Similarly, cell cycle proteins may be examined by Western blot analysis using commercially available antibodies. In a specific embodiment, the role of an ESP-regulator in cell cycle regulation is examined by contacting a cell with said ESP-regulator and comparing the cell cycle characteristics (such as cell cycle length, duration of phases, expression of cell cycle proteins etc.) to a cell which has not been contacted by the ESP-regulator. In another embodiment, cell populations are synchronized prior to detection

of a cell cycle protein. Cell cycle proteins may also be detected by FACS (fluorescence-activated cell sorter) analysis using antibodies against the protein of interest.

Further assays include but are not limited to detection of changes in length of the cell cycle or speed of cell cycle. In one embodiment the length of the cell cycle is determined by the doubling time of a population of cells. In another embodiment, FACS analysis is used to analyze the phase of cell cycle progression, or purify G1, S, and G2/M fractions (*see e.g.*, Delia, et al., 1997, *Oncogene* 14:2137-47). In another embodiment assays of this type are be performed on synchronized cell populations. In a further embodiment, cells are synchronized by a nocodazole block (Knehr et al., 1995, *Exp. Cell Res.* 217:546-553). In a further embodiment, length or speed of the cell cycle of a test population (such as BRCA1 mutant population) is compared to wildtype populations.

Lapse of cell cycle checkpoint(s), and/or induction of cell cycle checkpoint(s), may be examined by the methods described herein, or by any method known in the art. Without limitation, a cell cycle checkpoint is a mechanism which ensures that a certain cellular events occur in a particular order. Checkpoint genes are defined by mutations that allow late events to occur without prior completion of an early event (Weinert and Hartwell, 1993, *Genetics* 134:63-80). Induction or inhibition of cell cycle checkpoint genes may be assayed, for example, by Western blot analysis, or by immunostaining, etc. Lapse of cell cycle checkpoints may be further assessed by the progression of a cell thru the checkpoint without prior occurrence of specific events (e.g. progression into mitosis without complete replication of the genomic DNA).

In addition to the effects of expression of a particular cell cycle protein, activity and post-translational modifications of proteins involved in the cell cycle can play an integral role in the regulation and proliferative state of a cell. The invention provides for assays involved detected post-translational modifications (e.g. phosphorylation) by any method known in the art. For example, antibodies which detect phosphorylated tyrosine residues are commercially available, and may be used in western blot analysis to detect proteins with such modifications. In another example, modifications such as myristylation, may be detected on thin layer chromatography or reverse phase HPLC (*see e.g.*, Glover, 1988, *Biochem J.* 250:485-91; Paige, 1988, *Biochem J.* 250:485-91).

Activity of signaling and cell cycle proteins and/or protein complexes is often mediated by a kinase activity. In present invention provides for analysis of kinase activity by assays such as the histone H1 assay (*see e.g.*, Delia, et al., 1997, *Oncogene* 14:2137-47). In another embodiment, the kinase is a casein kinase. In one embodiment, the kinase assay is performed *in vitro*. In a further embodiment, the *in vitro* kinase assay is

performed with purified components. In a specific embodiment of the H1 Kinase Assay is performed as follows: cell lysates are precleaned by incubation in protein G(or A)-agarose. Lysates are immunoprecipitated and washed three times with immunoprecipitation buffer and twice with 1X kinase buffer (50 mM Tris-HCl 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT) without DDT. The kinase assay is then carried out on ice for 10 minutes in 35 μl of 1X kinase buffer containing 15 μCi of γ-32P ATP, 1.6 μg of histone H1, and 1.5 μM ATP. The kinase activities are measured by quantifying the intensities of histone-H1 phosphorylation using a PhosphorImager (Molecular Dynamics). In a preferred embodiment, the kinase assay experiments are repeated three times.

Other methods will be apparent to one skilled in the art and are within the scope of the invention.

5.6 ASSAYS FOR APOPTOSIS

Without limitation to a particular mechanism, programed cell death, or apoptosis is a process by which a cell actively undergoes death by the induction of a series of biochemical and morphological changes. Apoptosis plays an important role in development, homeostasis and disease (*see e.g.*, Lowe et al., 1993, *Cell* 74:957-967).

A variety of techniques known in the art may be used in the present invention to examine apoptosis. One assay for apoptosis is the Terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (*see e.g.* Lazebnik et al., 1994, *Nature* 371:346), by following the incorporation of fluorescein-dUTP (Yonehara et al., 1989, *J. Exp. Med.* 169:1747). Alternately, apoptosis signaling may be detected by ApoAlert™ staining (Gavrieli et al., 1992, *J. Cell. Biol.* 119:493). These assay kits are commercially available through suppliers such as Clontech and Boehringer Mannheim. Apoptosis may further be assayed by acridine orange incorporation (*see e.g.* Lucas et al., 1998, *Blood* 15:4730-41). Detection of a gene in the apoptotic pathway may be detected standard methods in the art such as by Western analysis using antibodies to the apoptotic gene of interest.

Additional assays for detection of apoptosis include detection of DNA fragmentation by DNA laddering on an agarose gel (*see e.g.*, Civielli et al., 1995, *Int. J. Cancer* 27:673-679; Yeung, 1998, *J. Biol. Chem.* 273:25198-202). In this technique, DNA fragmentation is detected on an agarose gel stained with ethidium bromide. Due to the specific sites at which DNA is fragmented during apoptosis, a characteristic pattern or

"ladder" results when the cellular DNA is examined by electrophoresis. Other methods will be readily apparent to one skilled in the art and are within the scope of the invention.

5.7 CELL ADHESION AND LOSS OF INVASIVENESS

A critical aspect of the formation of a metastatic cancer is the ability of a precancerous or cancerous cell to detach from primary site of disease, and establish a novel colony of growth at a secondary site. The ability of a cell to invade peripheral sites is reflective of a potential for a cancerous state. Invasion inhibition assays are within the scope of the invention. For example ESP-regulators may be assayed for their ability of inhibit invasion of a test cell. Loss of invasiveness may be measured by a variety of techniques known in the art including, for example, induction of E-cadherin-mediated cell-cell adhesion. Such E-cadherin-mediated adhesion can result in phenotypic reversion and loss of invasiveness (Hordijk et al., 1997, *Science* 278:1464-66).

Loss of invasiveness may further be examined by inhibition of cell migration. A variety of 2-dimensional and 3-dimensional cellular matrices are commercially available (Calbiochem-Novabiochem Corp. San Diego, CA). Cell migration across or into a matrix may be examined by microscopy, time-lapsed photography or videography, or any method in the art allowing measurement of cellular migration. In a related embodiment, loss of invasiveness is examined by response to hepatocyte growth factor (HGF). HGF-induced cell scattering is correlated with invasiveness of cells such as Mardin-Darby canine kidney (MDCK) cells. This assay identifies a cell population which has lost cell scattering activity in response to HGF (Hordijk et al., 1997, *Science* 278:1464-66).

Alternatively, invasion or loss of invasiveness may be measured by cell migration through a chemotaxis chamber (Neuroprobe/ Precision Biochemicals Inc. Vancouver , BC). In this assay, a chemo-attractant agent is incubated on one side of the chamber (e.g. the bottom chamber) and cells are plated on a filter separating the opposite side (e.g. the top chamber). In order for cells to pass from the top chamber to the bottom chamber, the cell must actively migrate through small pores in the filter. Checkerboard analysis of the number of cells which have migrated may then be correlated with invasivness (*see e.g.*, Ohnishi, 1993, *Biochem. Biophys. Res. Commun.*193:518-25).

5.8 HOST CELLS

The present invention delivery of ESP-regulators to primary cells, or cell lines for *in vitro* screening assay and *ex vivo* gene therapy. In accordance with the present

invention, a variety of primary or secondary cells or cell strains may be used including but not limited to cells isolated from breast, skin, bone marrow, liver, pancreas, kidney, adrenal and neurological tissue to name a few. Other cells types that may be used in accordance with the present invention are immune cells (such as T-cells, B-cells, natural killer cells, etc.), macrophages/monocytes, adipoctyes, pericytes, fibroblasts, neuronal cells, reticular cells etc. Breast cancer cell lines that may be used in accordance with the invention include but are not limited to UACC-893, UACC-812, ZR-75-1, ZR-75-30, MDA-MB-415, MDA-MB-415A, MDA-MB-435S, BT-483, and Hs 564(C).Mg. In a further embodiment, secondary cell lines may be used as engineered responsive cells and tissues in accordance with the present invention, including, but not limited to hepatic cell lines, such as CWSV. NR, Chang liver cells, or other cell lines such as CHO, VERO, BHK, Hela, COS, MDCK. 293, 373, CaSki and W138 cell lines.

5.9 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

As used herein, the novel compounds of the present invention, the compounds of the present compositions, and the compounds of the present methods are known collectively as "ESP-regulators". ESP-regulators include any compound that inhibits estrogen pathway signalling with the proviso that the ESP-regulator is not any of the chemotherapeutic or anti-cancer agents listed in Table 1. In a preferred embodiment the ESP-regulators inhibit the binding of p300 and/or CBP to the estrogen response enhancer with the proviso that the ESP-regulator is not any of the chemotherapeutic or anti-cancer agents listed in Table 1. In a most preferred embodiment the ESP-regulators inhibit transcription driven by estrogen response enhancers in cells having a mutation of BRCA1 in an essential domain (*e.g.*, the N-terminal RING finger domain, the consensus retinoblastoma binding motif and a domain in the C-terminal 550 amino acids) with the proviso that the ESP-regulator is not any of the chemotherapeutic or anti-cancer agents listed in Table 1.

Due to the activity of the ESP-regulator, the ESP-regulator are advantageously useful in veterinary and human medicine. For example, the ESP-regulator are useful for the treatment or prevention of cancer or neoplastic disease or inhibiting the growth of a cancer cell or neoplastic cell.

When administered to a subject, *e.g.*, an animal for veterinary use or to a human for clinical use, or when made to contact a cell or tissue, the ESP-regulator are preferably in isolated form. By "isolated" it is meant that prior to administration or contacting, a ESP-regulator is separated from other components of a synthetic organic

chemical reaction mixture or natural product source, *e.g.*, plant matter, tissue culture, bacterial broth, etc. Preferably, the ESP-regulator are isolated via conventional techniques, *e.g.*, extraction followed by chromatography, recrystalization, or another conventional technique. When in isolated form, the ESP-regulator are at least 90%, preferably at least 95%, of a single ESP-regulator by weight of that which is isolated.

The invention provides methods of treatment and prophylaxis by administration to a subject of an effective amount of a ESP-regulator. The subject is preferably an animal, including, but not limited, to an animal such a cow, horse, sheep, pig, chicken, turkey, quail, cat, dog, mouse, rat, rabbit, guinea pig, etc., and is more preferably a mammal, and most preferably a human.

The present compositions, which comprise one or more ESP-regulator, may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, etc., and can be used to administer a ESP-regulator of the invention. In certain embodiments, more than one ESP-regulator of the invention is administered to a subject. Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically to the ears, nose, eyes, or skin. The preferred mode of administration is left to the discretion of the practitioner, and will depend in-part upon the site of the medical condition (such as the site of cancer).

In specific embodiments, it may be desirable to administer one or more ESP-regulator of the invention locally to the area in need of treatment. This may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a cancer, tumor or neoplastic or pre-neoplastic tissue.

In certain embodiments, it may be desirable to introduce one or more ESP-regulator of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant. In certain embodiments, the ESP-regulator can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

In another embodiment, the ESP-regulator of the invention can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, *Science* 249:1527-1533; Treat et al.,1989, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365: Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the ESP-regulator can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, 1987, *CRC Crit. Ref. Biomed. Eng.* 14:201; Buchwald et al., 1980, *Surgery* 88:507; Saudek et al.,1989, *N. Engl. J. Med.* 321:574). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; *see also* Levy et al., 1985, *Science* 228:190; During et al., 1989, *Ann. Neurol.* 25:351; Howard et al., 1989, *J. Neurosurg.* 71:105). In yet another embodiment, a controlled-release system can be placed in proximity of the target of the ESP-regulator, *e.g.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, 1984, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138). Other controlled-release systems discussed in the review by Langer (1990, *Science* 249:1527-1533) may be used.

The present compositions will contain a therapeutically effective amount of a ESP-regulator, preferably in purified form, together with a suitable amount of a pharmaceutically acceptable carrier so as to provide the form for proper administration to the patient.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which a ESP-regulator is administered. Such pharmaceutical carriers can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea,

and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents may be used. When administered to a subject, the ESP-regulator and pharmaceutically acceptable carriers are preferably sterile. Water is a preferred carrier when the ESP-regulator is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

The present compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable carrier is a capsule (see *e.g.*, U.S. Patent No. 5,698,155). Other examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

In a preferred embodiment, the ESP-regulator are formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, ESP-regulator for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the compositions may also include a solubilizing agent. Compositions for intravenous administration may optionally include a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the ESP-regulator is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the ESP-regulator is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

Compositions for oral delivery may be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions may contain one or more optionally agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to

provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions may be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered ESP-regulator. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time delay material such as glycerol monostearate or glycerol stearate may also be used. Oral compositions can include standard carriers such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Such carriers are preferably of pharmaceutical grade.

The amount of the ESP-regulator that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the compositions will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Such animal models and systems are well known in the art.

The ESP-regulator of the invention are preferably assayed *in vitro*, and then *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays can be used to determine whether administration of a specific ESP-regulator or combination of ESP-regulator is preferred.

In one embodiment, a patient tissue sample is grown in culture, and contacted or otherwise administered with a ESP-regulator, and the effect of such ESP-regulator upon the tissue sample is observed and compared to a non-contacted tissue. In other embodiments, a cell culture model is used in which the cells of the cell culture are contacted or otherwise administered with a ESP-regulator, and the effect of such ESP-regulator upon the tissue sample is observed and compared to a non-contacted cell culture. Generally, a lower level of proliferation or survival of the contacted cells compared to the non-contracted cells indicates that the ESP-regulator is effective to treat a the patient. Such ESP-regulator may also be demonstrated effective and safe using animal model systems.

Other methods will be known to the skilled artisan and are within the scope of the invention.

5.10 INHIBITION OF CANCER AND NEOPLASTIC CELLS AND DISEASE

The ESP-regulator may be demonstrated to inhibit tumor cell proliferation, cell transformation and tumorigenesis *in vitro* and *in vivo* using a variety of assays known in the art, or described herein. Such assays may use cells of a cancer cell line, or cells from a patient. Many assays well-known in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring (^{3}H)-thymidine incorporation, by direct cell count, by detecting changes in transcription, translation or activity of known genes such as proto-oncogenes (*e.g.*, *fos, myc*) or cell cycle markers (Rb, cdc2, cyclin A, D1, D2, D3, E, etc). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as Western blotting or immunoprecipitation using commercially available antibodies (for example, many cell cycle marker antibodies are from Santa Cruz Inc.). mRNA can be quantitated by methods that are well known and routine in the art, for example by northern analysis, RNase protection, the polymerase chain reaction in connection with the reverse transcription, etc. Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in the art. Differentiation can be assessed visually based on changes in morphology, etc.

The present invention provides for cell cycle and cell proliferation analysis by a variety of techniques known in the art, including but not limited to the following:

As one example, bromodeoxyuridine (BRDU) incorporation may be used as an assay to identify proliferating cells. The BRDU assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly synthesized DNA. Newly synthesized DNA may then be detected using an anti-BRDU antibody (*see* Hoshino et al., 1986, *Int. J. Cancer* 38:369; Campana et al., 1988, *J. Immunol. Meth.* 107:79).

Cell proliferation may also be examined using (^{3}H)-thymidine incorporation (*see e.g.*, Chen, 1996, *Oncogene* 13:1395-403; Jeoung, 1995, *J. Biol. Chem.* 270:18367-73). This assay allows for quantitative characterization of S-phase DNA synthesis. In this assay, cells synthesizing DNA will incorporate (^{3}H)-thymidine into newly synthesized DNA. Incorporation may then be measured by standard techniques in the art such as by counting of radioisotope in a Scintillation counter (*e.g.* Beckman LS 3800 Liquid Scintillation Counter).

Detection of proliferating cell nuclear antigen (PCNA) may also be used to measure cell proliferation. PCNA is a 36 kilodalton protein whose expression is elevated in proliferating cells, particularly in early G1 and S phases of the cell cycle and therefore may serve as a marker for proliferating cells. Positive cells are identified by immunostaining using an anti-PCNA antibody (*see* Li et al., 1996. *Curr. Biol.* 6:189-199; Vassilev et al., 1995. *J. Cell Sci.* 108:1205-15).

Cell proliferation may be measured by counting samples of a cell population over time (*e.g.* daily cell counts). Cells may be counted using a hemacytometer and light microscopy (e.g. HyLite hemacytometer, Hausser Scientific). Cell number may be plotted against time in order to obtain a growth curve for the population of interest. In a preferred embodiment, cells counted by this method are first mixed with the dye Trypan-blue (Sigma), such that living cells exclude the dye, and are counted as viable members of the population.

DNA content and/or mitotic index of the cells may be measured, for example, based on the DNA ploidy value of the cell. For example, cells in the G1 phase of the cell cycle generally contain a 2N DNA ploidy value. Cells in which DNA has been replicated but have not progressed through mitosis (e.g. cells in S-phase) will exhibit a ploidy value higher than 2N and up to 4N DNA content. Ploidy value and cell-cycle kinetics may be further measured using propidium iodide assay (*see e.g.* Turner, et al., 1998, *Prostate* 34:175-81). Alternatively, the DNA ploidy may be determined by quantitation of DNA Feulgen staining (which binds to DNA in a stoichiometric manner) on a computerized microdensitometrystaining system (*see e.g.*, Bacus. 1989, *Am. J. Pathol.* 135:783-92). In an another embodiment, DNA content may be analyzed by preparation of a chromosomal spread (Zabalou, 1994, *Hereditas.* 120:127-40; Pardue, 1994, *Meth. Cell Biol.* 44:333-351).

The expression of cell-cycle proteins (e.g., CycA, CycB, CycE, CycD, cdc2, Cdk4/6, Rb, p21, p27, etc.) provide crucial information relating to the proliferative state of a cell or population of cells. For example, identification in an anti-proliferation signaling pathway may be indicated by the induction of $p21^{cip1}$. Increased levels of p21 expression in cells results in delayed entry into G1 of the cell cycle (Harper et al., 1993, *Cell* 75:805-816; Li et al., 1996, *Curr. Biol.* 6:189-199). p21 induction may be identified by immunostaining using a specific anti-p21 antibody available commercially (*e.g.* Santa Cruz). Similarly, cell-cycle proteins may be examined by Western blot analysis using commercially available antibodies. In another embodiment, cell populations are synchronized prior to detection of a cell cycle protein. Cell cycle proteins may also be detected by FACS (fluorescence-activated cell sorter) analysis using antibodies against the protein of interest.

Detection of changes in length of the cell cycle or speed of cell cycle may also be used to measure inhibition of cell proliferation by the ESP-regulator of the invention. In one embodiment the length of the cell cycle is determined by the doubling time of a population of cells (e.g., using cells contacted or not contacted with one or more ESP-regulator of the invention). In another embodiment, FACS analysis is used to analyze the phase of cell cycle progression, or purify G1, S, and G2/M fractions (*see e.g.*, Delia et al., 1997, *Oncogene* 14:2137-47).

Lapse of cell cycle checkpoint(s), and/or induction of cell cycle checkpoint(s), may be examined by the methods described herein, or by any method known in the art. Without limitation, a cell cycle checkpoint is a mechanism which ensures that a certain cellular events occur in a particular order. Checkpoint genes are defined by mutations that allow late events to occur without prior completion of an early event (Weinert and Hartwell, 1993, *Genetics* 134:63-80). Induction or inhibition of cell cycle checkpoint genes may be assayed, for example, by Western blot analysis, or by immunostaining, etc. Lapse of cell cycle checkpoints may be further assessed by the progression of a cell through the checkpoint without prior occurrence of specific events (e.g. progression into mitosis without complete replication of the genomic DNA).

In addition to the effects of expression of a particular cell cycle protein, activity and post-translational modifications of proteins involved in the cell cycle can play an integral role in the regulation and proliferative state of a cell. The invention provides for assays involved detected post-translational modifications (e.g. phosphorylation) by any method known in the art. For example, antibodies that detect phosphorylated tyrosine residues are commercially available, and may be used in Western blot analysis to detect proteins with such modifications. In another example, modifications such as myristylation, may be detected on thin layer chromatography or reverse phase HPLC (*see e.g.*, Glover, 1988, *Biochem. J.* 250:485-91; Paige, 1988, *Biochem J.* 250:485-91).

Activity of signaling and cell cycle proteins and/or protein complexes is often mediated by a kinase activity. The present invention provides for analysis of kinase activity by assays such as the histone H1 assay (*see e.g.*, Delia et al., 1997, *Oncogene* 14:2137-47).

The ESP-regulator can also be demonstrated to alter cell proliferation in cultured cells *in vitro* using methods which are well known in the art. Specific examples of cell culture models include, but are not limited to, for lung cancer, primary rat lung tumor cells (Swafford et al., 1997, *Mol. Cell. Biol.* 17:1366-1374) and large-cell undifferentiated cancer cell lines (Mabry et al., 1991, *Cancer Cells* 3:53-58); colorectal cell lines for colon

cancer (Park and Gazdar, 1996, *J. Cell Biochem. Suppl.* 24:131-141); multiple established cell lines for breast cancer (Hambly et al., 1997, *Breast Cancer Res. Treat.* 43:247-258; Gierthy et al., 1997, *Chemosphere* 34:1495-1505; Prasad and Church, 1997, *Biochem. Biophys. Res. Commun.* 232:14-19); a number of well-characterized cell models for prostate cancer (Webber et al., 1996, *Prostate, Part 1* 29:386-394; *Part 2* 30:58-64; and *Part 3* 30:136-142; Boulikas, 1997, *Anticancer Res.* 17:1471-1505); for genitourinary cancers, continuous human bladder cancer cell lines (Ribeiro et al., 1997, *Int. J. Radiat. Biol.* 72:11-20); organ cultures of transitional cell carcinomas (Booth et al., 1997, *Lab Invest.* 76:843-857) and rat progression models (Vet et al., 1997, *Biochim. Biophys Acta* 1360:39-44); and established cell lines for leukemias and lymphomas (Drexler, 1994, *Leuk. Res.* 18:919-927, Tohyama, 1997, *Int. J. Hematol.* 65:309-317).

The ESP-regulator can also be demonstrated to inhibit cell transformation (or progression to malignant phenotype) *in vitro*. In this embodiment, cells with a transformed cell phenotype are contacted with one or more ESP-regulator, and examined for change in characteristics associated with a transformed phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*), for example, but not limited to, colony formation in soft agar, a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, or expression of fetal antigens, etc. (see Luria et al., 1978, *General Virology*, 3d Ed., John Wiley & Sons, New York, pp. 436-446).

Loss of invasiveness or decreased adhesion may also be used to demonstrate the anti-cancer effects of the ESP-regulator. For example, a critical aspect of the formation of a metastatic cancer is the ability of a precancerous or cancerous cell to detach from primary site of disease and establish a novel colony of growth at a secondary site. The ability of a cell to invade peripheral sites is reflective of a potential for a cancerous state. Loss of invasiveness may be measured by a variety of techniques known in the art including, for example, induction of E-cadherin-mediated cell-cell adhesion. Such E-cadherin-mediated adhesion can result in phenotypic reversion and loss of invasiveness (Hordijk et al., 1997, *Science* 278:1464-66).

Loss of invasiveness may further be examined by inhibition of cell migration. A variety of 2-dimensional and 3-dimensional cellular matrices are commercially available (Calbiochem-Novabiochem Corp. San Diego, CA). Cell migration across or into a matrix may be examined by microscopy, time-lapsed photography or videography, or by any method in the art allowing measurement of cellular migration. In a

related embodiment, loss of invasiveness is examined by response to hepatocyte growth factor (HGF). HGF-induced cell scattering is correlated with invasiveness of cells such as Mardin-Darby canine kidney (MDCK) cells. This assay identifies a cell population that has lost cell scattering activity in response to HGF (Hordijk et al., 1997, *Science* 278:1464-66).

Alternatively, loss of invasiveness may be measured by cell migration through a chemotaxis chamber (Neuroprobe/ Precision Biochemicals Inc. Vancouver, BC). In such assay, a chemo-attractant agent is incubated on one side of the chamber (*e.g.*, the bottom chamber) and cells are plated on a filter separating the opposite side (*e.g.*, the top chamber). In order for cells to pass from the top chamber to the bottom chamber, the cells must actively migrate through small pores in the filter. Checkerboard analysis of the number of cells that have migrated may then be correlated with invasiveness (*see e.g.*, Ohnishi, 1993, *Biochem. Biophys. Res. Commun.* 193:518-25).

The ESP-regulator can also be demonstrated to inhibit tumor formation *in vivo*. A vast number of animal models of hyperproliferative disorders, including tumorigenesis and metastatic spread, are known in the art (see Table 317-1, Chapter 317, "Principals of Neoplasia," in *Harrison's Principals of Internal Medicine, 13th Edition,* Isselbacher et al., eds., McGraw-Hill, New York, p. 1814; and Lovejoy et al., 1997, *J. Pathol.* 181:130-135). Specific examples include for lung cancer, transplantation of tumor nodules into rats (Wang et al., 1997, *Ann. Thorac. Surg.* 64:216-219) or establishment of lung cancer metastases in SCID mice depleted of NK cells (Yono and Sone, 1997, *Gan To Kagaku Ryoho* 24:489-494); for colon cancer, colon cancer transplantation of human colon cancer cells into nude mice (Gutman and Fidler, 1995, *World J. Surg.* 19:226-234), the cotton top tamarin model of human ulcerative colitis (Warren, 1996, *Aliment. Pharmacol. Ther.* 10 Suppl 2:45-47) and mouse models with mutations of the adenomatous polyposis tumor suppressor (Polakis, 1997, *Biochim. Biophys. Acta* 1332:F127-F147); for breast cancer, transgenic models of breast cancer (Dankort and Muller, 1996, *Cancer Treat. Res.* 83:71-88; Amundadittir et al., 1996, *Breast Cancer Res. Treat.* 39:119-135) and chemical induction of tumors in rats (Russo and Russo, 1996, *Breast Cancer Res. Treat.* 39:7-20); for prostate cancer, chemically-induced and transgenic rodent models, and human xenograft models (Royai et al., 1996, *Semin. Oncol.* 23:35-40); for genitourinary cancers, induced bladder neoplasm in rats and mice (Oyasu, 1995, *Food Chem. Toxicol* 33:747-755) and xenografts of human transitional cell carcinomas into nude rats (Jarrett et al., 1995, *J. Endourol.* 9:1-7); and for hematopoietic cancers, transplanted allogeneic marrow in animals (Appelbaum, 1997, *Leukemia* 11 Suppl. 4:S15-S17). Further, general animal models applicable to many types of cancer have been described, including, but not restricted to, the

p53-deficient mouse model (Donehower, 1996, *Semin. Cancer Biol.* 7:269-278), the Min mouse (Shoemaker et al., 1997, *Biochem. Biophys. Acta* 1332:F25-F48), and immune responses to tumors in rat (Frey, 1997, *Methods* 12:173-188).

For example, a ESP-regulator can be administered to a test animal, preferably a test animal predisposed to develop a type of tumor, and the test animal subsequently examined for an decreased incidence of tumor formation in comparison with controls not administered the ESP-regulator. Alternatively, a ESP-regulator can be administered to test animals having tumors (*e.g.*, animals in which tumors have been induced by introduction of malignant, neoplastic, or transformed cells, or by administration of a carcinogen) and subsequently examining the tumors in the test animals for tumor regression in comparison to controls not administered the ESP-regulator.

5.11 TREATMENT OR PREVENTION OF CANCER OR A NEOPLASTIC DISEASE IN COMBINATION WITH CHEMOTHERAPY OR RADIOTHERAPY

Cancer or a neoplastic disease, including, but not limited to, neoplasms, tumors, metastases, or any disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration of a composition comprising a pharmaceutically acceptable carrier and a ESP-regulator compound. The compositions can comprise one or more ESP-regulator compounds, or a pharmaceutically acceptable salt thereof.

In certain embodiments, one or more ESP-regulator of the invention are used to treat or prevent cancer or neoplastic disease in combination with one or more anti-cancer, chemotherapeutic agents including, but not limited to, methotrexate, taxol, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbizine, etoposides, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel. In a preferred embodiment, one or more ESP-regulator of the invention is used to treat or prevent cancer or neoplastic disease in combination with one or more chemotherapeutic or other anti-cancer agents including, but not limited to those presented in Table 1.

TABLE 1 CHEMOTHERAPEUTICS AND OTHER ANTI-CANCER AGENTS

Radiation:	γ-radiation

<u>Alkylating agents</u>	
Nitrogen mustards:	cyclophosphamide
	Ifosfamide
	trofosfamide
	Chlorambucil
Nitrosoureas:	carmustine (BCNU)
	Lomustine (CCNU)
Alkylsulphonates	busulfan
	Treosulfan
Triazenes:	Dacarbazine
Platinum containing compounds:	Cisplatin
	carboplatin
<u>Plant Alkaloids</u>	
Vinca alkaloids:	vincristine
	Vinblastine
	Vindesine
	Vinorelbine
Taxoids:	paclitaxel
	Docetaxol
<u>DNA Topoisomerase Inhibitors</u>	
Epipodophyllins:	etoposide
	Teniposide
	Topotecan
	9-aminocamptothecin
	campto irinotecan
	crisnatol
<u>mytomycins</u>:	
mytomycin C	Mytomycin C
<u>Anti-metabolites</u>	
<u>Anti-folates</u>:	
DHFR inhibitors:	methotrexate
	Trimetrexate
IMP dehydrogenase Inhibitors:	mycophenolic acid

	Tiazofurin
	Ribavirin
	EICAR
Ribonuclotide reductase Inhibitors:	hydroxyurea
	deferoxamine
<u>Pyrimidine analogs</u>:	
Uracil analogs	5-Fluorouracil
	Floxuridine
	Doxifluridine
	Ratitrexed
Cytosine analogs	cytarabine (ara C)
	Cytosine arabinoside
	fludarabine
<u>Purine analogs</u>:	mercaptopurine
	Thioguanine
<u>Hormonal therapies</u>:	
Receptor antagonists:	
Anti-estrogens	Tamoxifen
	Raloxifene
	megestrol
LHRH agonists:	goscrclin
	Leuprolide acetate
Anti-androgens:	flutamide
	bicalutamide
<u>Retinoids/Deltoids</u>	
Vitamin D3 analogs:	EB 1089
	CB 1093
	KH 1060
<u>Photodyamic therapies</u>:	vertoporfin (BPD-MA)
	Phthalocyanine

	photosensitizer Pc4
	Demethoxy-hypocrellin A
	(2BA-2-DMHA)
Cytokines:	Interferon-α
	Interferon-γ
	Tumor necrosis factor
Others:	
Isoprenylation inhibitors:	Lovastatin
Dopaminergic neurotoxins:	1-methyl-4-phenylpyridinium ion
Cell cycle inhibitors:	staurosporine
Actinomycins:	Actinomycin D
	Dactinomycin
Bleomycins:	bleomycin A2
	Bleomycin B2
	Peplomycin
Anthracyclines:	daunorubicin
	Doxorubicin (adriamycin)
	Idarubicin
	Epirubicin
	Pirarubicin
	Zorubicin
	Mitoxantrone
MDR inhibitors:	verapamil
Ca^{2+}ATPase inhibitors:	thapsigargin

In other embodiments, a composition comprising one or more ESP-regulator is administered along with radiation therapy and/or with one or a combination of chemotherapeutic agents, preferably with one or more chemotherapeutic agents with which

treatment of the cancer has not been found to be refractory. The ESP-regulator can be administered to a patient that has also undergone surgery as treatment for the cancer.

In another specific embodiment, the invention provides a method to treat or prevent cancer that has shown to be refractory to treatment with a chemotherapy and/or radiation therapy.

In a specific embodiment, a composition comprising one or more ESP-regulator is administered concurrently with chemotherapy or radiation therapy. In another specific embodiment, chemotherapy or radiation therapy is administered prior or subsequent to administration of a present composition, preferably at least an hour, five hours, 12 hours, a day, a week, a month, more preferably several months (*e.g.*, up to three months), subsequent to administration of a therapeutic of the invention.

The chemotherapy or radiation therapy administered concurrently with, or prior or subsequent to, the administration of a present composition can be accomplished by any method known in the art. The chemotherapeutic agents are preferably administered in a series of sessions, any one or a combination of the chemotherapeutic agents listed above can be administered. With respect to radiation therapy, any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater that 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used for skin cancers. Gamma-ray emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements, may also be administered to expose tissues to radiation.

Additionally, the invention provides methods of treatment of cancer or neoplastic disease with a present composition as an alternative to chemotherapy or radiation therapy where the chemotherapy or the radiation therapy has proven or may prove too toxic, *e.g.*, results in unacceptable or unbearable side effects, for the subject being treated. The subject being treated with the present compositions may, optionally, be treated with other cancer treatments such as surgery, radiation therapy or chemotherapy, depending on which treatment is found to be acceptable or bearable.

5.12 CANCER AND NEOPLASTIC DISEASE TREATABLE OR PREVENTABLE

Cancers or neoplastic diseases and related disorders that can be treated or prevented by administration of the present compositions include but are not limited to those

listed in Table 2 (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia):

TABLE 2CANCERS AND NEOPLASTIC DISORDERS

- Leukemia
  - acute leukemia
  - acute lymphocytic leukemia
  - acute myelocytic leukemia
    - myeloblastic
    - promyelocytic
    - myelomonocytic
    - monocytic
    - erythroleukemia
  - chronic leukemia
  - chronic myelocytic (granulocytic) leukemia
  - chronic lymphocytic leukemia
- Polycythemia vera
- Lymphoma
  - Hodgkin's disease
  - non-Hodgkin's disease
- Multiple myeloma
- Waldenström's macroglobulinemia
- Heavy chain disease
- Solid tumors
  - sarcomas and carcinomas
    - fibrosarcoma
    - myxosarcoma
    - liposarcoma
    - chondrosarcoma
    - osteogenic sarcoma
    - chordoma
    - angiosarcoma
    - endotheliosarcoma
    - lymphangiosarcoma
    - lymphangioendotheliosarcoma
    - synovioma
    - mesothelioma
    - Ewing's tumor
    - leiomyosarcoma
    - rhabdomyosarcoma
    - colon carcinoma
    - pancreatic cancer
    - breast cancer
    - ovarian cancer
    - prostate cancer
    - squamous cell carcinoma
    - basal cell carcinoma
    - adenocarcinoma

sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
bladder carcinoma
epithelial carcinoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
meningioma
melanoma
neuroblastoma
retinoblastoma

In specific embodiments. cancer. malignancy or dysproliferative changes (such as metaplasias and dysplasias). or hyperproliferative disorders. are treated or prevented in the ovary, breast. colon. lung. skin. pancreas. prostate. bladder. or uterus. In other specific embodiments. sarcoma. melanoma. or leukemia is treated or prevented.

In a preferred embodiment the present compositions are used to treat or prevent cancers in which the cancer cells express the estrogen receptor.

In a highly preferred embodiment. the present compositions are used to treat or prevent cancers including those of the breast or prostate.

In specific embodiments of the invention, the present compositions are used to inhibit the growth of a cell. said cell being derived from a cancer or neoplasm in Table 2 or herein.

5.13 KITS

The invention further provides kits that facilitate the use of ESP-regulators described herein. The kits described herein may be conveniently used, *e.g.*, in clinical settings to treat a patient suffering from a disorder involving inappropriate cell proliferation.

The kits comprise one or more containers filled with one or more ESP-regulators of the invention. In certain preferred embodiments, e.g., when administered to a patient to treat cancer, the kit may also contain one or more other chemotherapeutic agents useful for inhibiting cancer to be administered in combination with the ESP-regulators of the invention. In various other embodiments, the kit can also comprise, *e.g.*, a buffering agent, a preservative, or a stabilizing agent. The kit can also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Optionally associated with such container(s) can be instructions for use of the kit as a treatment along with a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6. EXAMPLES

6.1 BRCA1 GENE TO MODULATE THE TRANSCRIPTIONAL ACTIVITY OF ER-α

To assess the effect of BRCA1 on estrogen response, we measured the ability of the BRCA1 gene to modulate the transcriptional activity of ER-α in transient transfection assays. Wild-type BRCA1 expression plasmid (wtBRCA1) was created by cloning BRCA1 cDNA into the pcDNA3 vector (Invitrogen) using artificially engineered 5' Hind III and 3' Not I sites. Cells were assayed for stimulation of ERE-TK-Luc reporter activity by 17β-estradiol (E2). ERE-TK-Luc is composed of the vitellogenin A2 estrogen-responsive enhancer (ERE) controlling a minimal thymidine kinase promoter (TK81) that is operably linked to the luciferase gene, in plasmid pGL2 (Henttu et al., 1997, *Mol. Cell. Biol.* 17:1832). Proliferating cells at 50-70% of confluency in 24-well dishes were incubated overnight with 0.5 μg of each vector (unless otherwise indicated) in serum-free DMEM containing Lipofectin (Life Technologies). The total transfected DNA was kept constant by addition of control vector. Cells were washed, incubated in serum-free phenolphthalein-free DMEM (0.2 ml per well) ± 17β-estradiol (E2, 1 μM) for 24 hr. and harvested for luciferase assays.

Human prostate cancer cell line DU-145 lacks ER-α; but when supplied with exogenous ER-α, these cells became competent for estrogenic signalling, as demonstrated

by estradiol (E2) stimulated activation of estrogen-responsive reporter ERE-TK-Luc (Fig. 1a). Equally strong inhibition of ER-α signalling by wtBRCA1 was observed in breast cancer cell line T47D, in two other human breast cancer cell lines (MCF-7 and MDA-MB-231), and in two other human prostate cancer cell lines (LNCaP and TsuPr-1) (Figs. 1a and 1b). However, three cervical cancer cell lines showed relatively weak inhibition of ER-α signalling by wtBRCA1: E2-stimulated reporter activity in the presence of wtBRCA1 was about 40-50% of that in the presence of empty vector (Fig. 1b).

Mutations of BRCA1 cause an increased risk for breast, ovarian, and prostatic cancers (Mild et al., 1994, *Science* 266:66-71; Streuwing, 1997, *New Engl J Med* 336:1401-1408; Gayther et al., 1995, *Nat Genet* 11:428433). We tested a panel of mutant BRCA1 genes, including tumor-associated and artificially engineered mutants (Fig. 1c), to assess the impact of mutations on the ability of BRCA1 to inhibit ER-α activity.

Mutant BRCA1 vectors were created by modification of the BRCA1 cDNA in the pcDNA3 vector. BRCA1 mutants 5382insC and C53655G were a gift from Dr. Barbara Weber. Expression of the BRCA1 genes were confirmed by semi-quantitative RT-PCR, using three sets of primers mapping to different sites in BRCA1 (Fig. 1d). Semi-quantitative RT-PCR assays were performed as described earlier (Fan et al, 1998,. *Oncogene* 16:3069-3083). The BRCA1 primer sequences (5' → 3') and the locations within the cDNA sequence (GenBank accession number U15595) were as follows: primer #1, sense TTG CGG GAG GAA AAT GGG TAG TTA, antisense GAA GTA GTA AGT GGG AAC CGT GT (bp 5239 to 5525, 287-bp product); primer #2, sense GAG TAA CAA GCC AAA TGA ACA G, antisense TGG ATA CTT AAA GCC TTC TGT G (bp 2159 to 2616, 457-bp product); and primer #3, sense AAG GAA CCT GTC TCC ACA AAG T, antisense GTC AAG CTG AAA AGC ACA AAT G (bp 213 to 408, 196-bp product). The primers for the control gene β-actin were: sense TTG TTA CCA ACT GGG GAC GAT A, antisense TCG TCC TTC TAG TTC TAG (bp 265 to 1028, 764-bp) All three primers gave amplified products of the expected sizes. Primer #1 yielded increased levels of the 287-bp product in cells transfected with wtBRCA1, 5677insA, RXRXH, T300G, C5365G, and 5382insC; but mutations that truncate the BRCA1 mRNA 5' to the primer (ΔBamHI, ΔKpnI, ΔEcoRI) failed to yield an increase in the 287-bp product, relative to untransfected or empty vector-transfected controls. Primer #2 yielded an increase in the 457-bp product in cells transfected with wtBRCA1, 5677insA, ΔBamHI, RXRXH, T300G, 5382insC, and C5365G, all of which yield mRNAs that include the amplified cDNA segment. On the other hand, ΔKpnI and ΔEcoRI, which encode mRNAs truncated 5' to the primer, failed to yield an increase in the 457-bp product. Primer #3, which maps to the amino-terminus of BRCA1,

gave increased 196-bp product in all but control transfections. These findings confirm the expression of all BRCA1 mRNAs, except l85delAG, which is not detected because it is truncated 5' to the primers. Because of the tiny size of the predicted product (38 aa), l85deLAG is likely to act as a null mutation.

We tested the ability of the BRCA1 cDNAs to inhibit estradiol (E2) induced, ER-α mediated ERE-TK-Luc reporter activation in DU-145 and T47D cells. BRCA1-5677insA (Y1853term), which corresponds to a tumor-associated mutation that deletes 11 aa and ablates activity of the carboxyl-terminal transactivation domain (TAD) (Monteiro et al., 1996, *Proc Natl Acad Sci USA* 93:13595-13599), abolished reporter activity (Fig. 1e). However, two other carboxyl-terminal tumor mutations, 5382insC (Ql756term) and C5365G (P1749R), failed to inhibit ER-α activity. These mutations also abolish the activity of the TAD.

Since the tumor-associated BRCA1 mutant 5677insA strongly suppressed ER-α activity at the single plasmid dose tested, we compared the relative ability of 5677insA vs wtBRCA1 to inhibit ER-α at a series of different plasmid doses. When tested side-by-side, the wtBRCA1 gene was about 5-fold more effective (μg/μg) than the 5677insA gene at inhibiting ER-α transcriptional activity in DU-145 and T47D cells (Fig. 1f), suggesting that the 5677insA mutation significantly weakens the ability of BRCA1 to inhibit ER-α activity. Taken together, these results suggest that a site within the minimal TAD of BRCA1 (last 100-120 aa) is required for the inhibition of ER-α activity.

A tumor-associated mutation of the amino-terminal RING domain (T300G) and a mutation of the retinoblastoma binding motif LXCXE (RXRXH) abolished ER-α inhibitory activity in both cell lines (Fig 1e). A second tumor-associated point mutation of the RING domain (T31OG) also failed to suppress ER-α activity (data not shown). Examination of deletion mutants revealed that 185deAG, a tumor-associated mutation that truncates BRCA1 in the RING domain, and BRCA1 ΔEcoRI, which truncates BRCA1 just before LXCXE, failed to inhibit ER-α activity (Fig. 1e). Mutants that truncate BRCA1 after LXCXE but before the TAD (ΔKpnI, ΔBamHI) yielded 50% inhibition in DU-145 and no inhibition in T47D. Since E2 typically induces reporter activity by (100-1000)-fold and wtBRCA1 reduces the activity to near baseline (one-fold stimulation), a 50% reduction of activity is probably not meaningful. Differences in the degree of inhibition of ER-α activity by different BRCA1 transgenes could not be attributed to differences in transfection efficiency, since similar results were obtained when a control plasmid (pRSV-β-gal) was co-transfected and reporter activity was normalized by β-galactosidase (data not shown). These findings suggest that inhibition of ER-α requires several functional domains, including the

RING and LXCXE motifs, as well as another domain located carboxyl-terminal to the BamHI site (aa 1313).

These experiments document the ability of BRCA1 genes to inhibit an artificial E2-dependent reporter gene. We studied two endogenous E2-responsive breast cancer cell lines (MCF-7 and T47D) to determine if the wtBRCA1 gene could inhibit the physiologic expression of two endogenous E2-responsive genes, pS2 and cathepsin D (Jeltsch et al., 1987, *Nucleic Acids Res* 15:1401-1414; Faust et al., *Proc Natl Acad Sci USA* 82: 4910-4914). Exposure to E2 (1 μM) for 24 hr induced significant increases in the production of pS2 and cathepsin D proteins as evidenced by Western blot analysis (anti-pS2= V3030, mouse monoclonal, Biomeda Corp., 1:100 and anti-cathepsin D= 06-467, rabbit polyclonal, Upstate Biotechnol., 1:200). This increase was abrogated or reduced in cells transfected with wtBRCA1, as compared with the pcDNA3 vector (Fig. lg). The E2-induced stimulation of pS2 expression and its inhibition by the wtBRCA1 gene was also observed at the mRNA level, by Northern blotting (not shown). In these experiments, the wtBRCA1 gene did not usually reduce pS2 or cathepsin D expression to below the unstimulated control levels.

These results demonstrate that BRCA1 is a specific repressor of transcription. Thus, the discovery of the invention indicates that the regulation of the estrogen receptor activity plays an important role in the development of cancers associated with BRCA1 mutation. The present invention discloses a novel use for the regulation of the estrogen receptor activity and signalling in the treatment or prevention of cancers or disease associated with BRCA1 mutation.

6.2 BRCA1 AND ESTROGEN RECEPTOR INTERACTING DOMAINS

To determine if a physical association of BRCA1 and ER-α night contribute to suppression of ER-α activity, we tested an ER positive breast cancer cell line (MCF-7) by immunoprecipitation (IP)-Western blotting. Subconfluent proliferating cells in 150 cm^2 dishes were harvested, and nuclear extracts were prepared (Dignam et al., 1983, *Nucleic Acids Res* 5:1475-1489). Each IP was carried out using 6 μg of antibody or antibody combination and 1000 μg of nuclear extract. The IP antibodies were as follows: BRCA1 (Ab-1 + Ab-2 + Ab-3, Oncogene Research Products), ER-α (mouse monoclonal, catalog #17529, Upstate Biotechnology), and control (normal mouse IgG). Precipitated proteins were collected, washed, and boiled in Laemmeli sample buffer. These samples were then subjected to Western blotting. Equal aliquots of total protein (100 μg per lane) were

electrophoresed on a 4-13% SDS-polyacrylamide gradient gel, transferred to nitrocellulose membranes (Millipore), and blotted using as primary antibodies: BRCA1 (C-20, rabbit polyclonal, Santa Cruz Biotechnology, 1:200 dilution), ER-α (11-184, rabbit polyclonal, Santa Cruz, 1:1000). Proteins were visualized using the enhanced chemiluminescence system, with colored markers (BioRad) as size standards.

ER-α was detected in the BRCA1 IP of MCF-7 cell nuclei and vice versa (Fig. 2a). Control IPs using non-immune antibodies failed to yield BRCA1 or ER-α protein bands. While BRCA1 was precipitated from DU-145 cells, there was little or no ER-α detected in the ER-α or BRCA1 IPs (Fig. 2a), consistent with the ER negative status of this cell line (Webber et al., 1997, *The Prostate* 30:58-64). On the other hand, DU-145 cells transfected with wtBRCA1 (to enhance expression) and ER-α expression vectors showed association of BRCA1 and ER-α, in the presence or absence of E2 (Fig. 2b). These findings indicate that BRCA1 and ER-α associate within the same protein complex, independently of estrogen.

To determine if the association of BRCA1 and ER-α is due to a direct physical interaction, we performed GST capture assays using *in vitro* translated (IVT) BRCA1 and beads coated with a series of GST-ER fusion proteins. GST bead assays were performed essentially as described before (Wang et al., 1993, *Science* 260:1330-1335). ^{35}S-methionine-labeled proteins were prepared by *in vitro* transcription (using the T7 promoter of pcDNA3) and translation. The GST fusion proteins were generated from cDNAs cloned into the pGEX vector, expressed in E. coli, and purified by affinity chromatography (Webb P et al., 1998, *Mol Endocrinol* 12:1605-1618). The GST-BRCA1 304-1863 vector was provided by Dr. Barbara Weber. Labeled proteins were incubated with GST (control) or GST fusion proteins for 4 hr at 4°C, recovered using GST agarose beads, eluted in boiling sample buffer, and analyzed by SDS-PAGE autoradiography. GST fusion proteins were visualized by Western blotting, using an anti-GST mouse monoclonal (B-14, Santa Cruz, 1:10,000).

Fig. 3a shows schematic diagrams of the series of ER-α constructs tested; Fig. 3b shows the various GST-ER fusion proteins visualized by Western blotting, using an anti-GST antibody. Among six GST-ER proteins tested, three carboxyl-terminal ER-α peptides (ER 282-420, ER 282-595, ER 393-595) captured IVT-wtBRCA1; while three more amino-terminal ER-α peptides (ER 1-185, ER 1-282, ER 282-337) failed to capture wtBRCA1 (Fig. 3c).

To delineate the binding site(s) for ER-α on BRCA1, we tested various IVT BRCA1 proteins (see Fig. 1c) for binding to GST-ER 282-420. An autoradiograph of the

IVT BRCA1 translation products is shown in Fig. 3d, which illustrates the relative translational efficiency for the different products. All BRCA1 proteins tested bound to GST-ER 282-420, with the most binding for BRCA1ΔEcoRI (Fig. 3e). The ratio of bound:input BRCA1 was higher for carboxyl-terminal peptides than for full-length or near full-length BRCA1 proteins (Fig. 3f), suggesting interference to binding by a carboxyl-terminal portion of BRCA1, but this remains to be proven. Finally, a fusion protein of GST and N-terminally deleted BRCA1 (GST-BRCA1 304-1863) failed to pull down IVT ER-α (data not shown). These findings suggest an E2-independent interaction of BRCA1 and ER-α involving aa 1-302 of BRCA1 and the AF-2/LBD region of ER-α.

The failure of the BRCA1ΔEcoRI gene to inhibit ER-α activity suggests that binding to ER-α is insufficient for inhibition and that a more carboxyl-terminal domain of BRCA1 is also required. However, co-transfection of BRCA1ΔEcoRI resulted in a dose-dependent rescue of the wtBRCA1-mediated inhibition of ER-α activity (Fig. 2c), suggesting that an amino-terminal fragment of BRCA1 (aa 1-302) can function as a dominant inhibitor of the full-length wtBRCA1.

6.3 EFFECT OF BRCA1 ON PROTEINS IN THE ESTROGEN RECEPTOR COACTIVATING COMPLEX

DU-145 cells stably expressing wtBRCA1 or 5677insA showed decreased levels of the transcriptional adaptor p300 (Fan et al., 1998, Oncogene 16:3069-3083). p300 and its homolog CBP (CREB binding protein) are components of the ER co-activator complex (Hanstein et al., 1996, *Proc Natl Acad Sci USA* 93:11540-11545; Smith et al., 1996, *Proc Natl Acad Sci USA* 93:8884-8885; Kamei et al., 1996, *Cell* 85:403-414). Therefore, we examined the ability of different BRCA1 genes to regulate their expression. Cells were lysed and extracts were Western blotted as in Section 6.2 (anti-p300 = 05-267, mouse monoclonal, Upstate Biotechnology, 4 μg/ml: anti-CBP =C-20, rabbit polyclonal, Santa Cruz, 1:400).

wtBRCA1 and 5677insA caused down-regulation of p300 at 24 hr post transfection in both DU-145 and T47D cells: while mutant BRCA1 genes that gave partial or no inhibition of ER-α activity (T300G, RXRXH, ΔKpnI, ΔEcoRI, 185delAG, 5382insC, C5365G) had no effect on p300 protein levels (Fig. 4a & b). The down-regulation of p300 by wtBRCA1 and 5677insA was also observed at the mRNA level and was not reversed by proteasomal inhibitors (data not shown). None of the BRCA1 genes caused a reduction of CBP in either cell type (Fig. 4a & b). In contrast, wtBRCA1 had no effect on p300 (or CBP) levels in three cervix cancer cell lines (Fig. 4c), consistent with the relatively weak

inhibition of ER-α activity by BRCA1 in this cell type (Fan et al., 1999, *Science* 284:1354-1356).

We reported that DU-145 cells transfected with wt BRCA1 or BRCA1-5677insA showed decreased expression of the transcriptional adaptor p300, compared to parental and vector transfected cells (Fan et al., 1998, *Oncogene* 16:3069). p300 and its functional homolog CBP (CREB binding protein) are components of the ER-α coactivator complex required for full transcriptional activation of ER-α (Kamei et al., 1996, *Cell* 85:403, Hanstein et al, 1996., *Proc. Natl. Acad. Sci. U.S.A.* 93:11540; Smith et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93:8884). p300 interacts with ER-α and steroid receptor coactivator-1 (SCR-1) via – and C-terminal domains, respectively, and may link ER-α to the basal transcriptional machinery via its E1A/TFIIB binding domain, which also binds p300/CBP-associated factor PCAF and transcription factors c-Jun and c-Fos (Kamei et al., 1996, *Cell* 85:403; Hanstein et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93:11540; Smith et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93:8884; Wang et al., 1997, *Mol. Cell. Biol.* 17:519). Thus, the p300, PCAF, and CBP, or derivatives or mutants thereof, may act as ESP-regulators of the invention.

6.4 REGIONS OF THE ESTROGEN RECEPTOR COACTIVATING COMPLEX IMPORTANT FOR INTERACTION WITH BRCA1 AND ESTROGEN RECEPTOR

We tested the effects of wild-type (wt-p300) and mutant p300 expression vectors (Fig. 5a) on wtBRCA1 inhibition of ER-α activity. In the absence of wtBRCA1, wt-p300 and several mutant p300 genes caused a moderate (up to a 2 or 3-fold) increase in E2-stimulated ER-α/ERE-TK-Luc reporter activity. Co-transfection of wt-p300 rescued the wtBRCA1 inhibition of reporter activity in both DU-145 and T47D cells (Fig. 5b). p300 mutants missing the histone acetyl transferase (HAT) or Bromo domain also rescued the inhibition: but a mutant missing the E1A/TFIIB binding site, corresponding to cysteine/histidine rich domain CH3, failed to rescue the inhibition.

Interestingly, a p300 mutant containing only CH3 and surrounding sequences (p300 1514-1922) fully rescued the inhibition, suggesting that the E1A/TFIIB site is essential for the rescue. A wt-CBP expression vector also rescued the wtBRCA1 inhibition of ER-α in both cell types (Fig. 5c), indicating that p300 and CBP are similar with regard to the rescue function. In contrast top300 and CBP, expression vectors for two other nuclear receptor co-activators, PCAF (p300/CBP-associated co-factor) (Wang et al., 1997, *Mol Cell Biol* 17:519-527) and GRIP 1 (glucocorticoid receptor-interacting protein) (Ding et al.,

1998, *Mol Endocrinol* 12:302-313) failed to rescue the wtBRCA1 inhibition of ER-α activity (data not shown). Co-transfection of wt-p300 but not p300 1514-1922 blocked the *wtBRCA1*-mediated down-regulation of p300 levels (Fig. 5*d*). The finding that p300 1514-1922 rescues the *wtBRCA1* inhibition of ER-α activity but does not restore the levels of endogenous p300 suggests that the p300 rescue is distinct from its co-activator function.

We performed GST capture assays to delineate the interaction between p300/CBP and ER-α. These assays revealed an E2-independent interaction between the AF-2/LBD region of ER-α and the carboxyl-terminal, but not the amino-terminal, region of CBP (aa 1680-2441) (Fig. 6a). As a positive control, GRIP1 exhibited E2-dependent binding to ER-α AF-2/LBD. Fig. 6b shows the binding of ER-α and other proteins to a series of overlapping fragments of the carboxyl-terminal region of CBP. The ER-α binding site of CBP mapped to the CH3 domain, the region found to be essential for rescue of the wtBRCA1 inhibition of ER-α activity. The CH3 domain also interacted with c-Fos and TFIIB (positive controls) but not PR-B (progesterone receptor-B) or GRIP1; and the binding of ER-α to the two overlapping CBP fragments containing the CH3 region was E2-independent (Fig. 6c). Lastly, we showed that the p300 1514-1922 fragment, which contains the CH3 region, interacts with the same region of ER-α (aa 282-420) that binds wtBRCA1. And preincubation of GST-ER 282-420 with IVT p300 1514-1922 or wtBRCA1 competed out the binding of IVT wtBRCA1 or p300 1514-1922, respectively (Fig. 6d). The GST competition assays were performed as described in Section 6.2, except that GST-ER 282-420 was pre-incubated for 3 hr at 4°C with different quantities of IVT competing protein.

These results suggest that other mechanisms besides the down-regulation of p300/CBP may contribute to inhibition of ER-α activity by BRCA1. Thus, the invention provides inhibition of the estrogen signalling pathway (e.g., via ER-α.) by one or more molecules. BRCA1 is linked to the RNA polymerase II holoenzyme via its C-terminal TAD: and p300 and CBP are also present within the RNA polymerase II complex (Scully et al., 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:5605; Neish et al., 1998, *Nucleic Acids Res.* 26:847). An alternatively spliced form of BRCA1 may interact with CBP *in vivo* and *in vitro*, via the CH3 domain of CBP (Cui et al., 1998, *Oncol. Rep.* 5:591). Further, BRCA1 (through an N-terminal domain) interacts with p53 and modulates p53 transcriptional activity (Zhang et al., 1998, *Oncogene* 16:1713; Ouchi et al., 1998, *Proc. Natl. Acad. Sci. U.S.A.* 95:2302).

The present invention indicates that BRCA1 may function, in part, as an adaptor protein, with negative or positive transcriptional regulatory activity and such regulation is useful in the treatment and prevention of disease associated with BRCA1 mutation.

The present invention demonstrates that regardless of mechanism, failure to repress ER-α activity contributes to breast cancer in BRCA1 mutation carriers. This discovery is consistent with the finding that 8/9 mutant BRCA1 genes (including four tumor-associated mutations (5382insC, C5365G, T300G, 185delAG), a mutation of the LXCXE site, and three deletion mutants) showed decreased or no ER-α repressor activity. Two of these mutations, 5382insC and 185delAG, are very common and occur in 0.3% and 1%, respectively, of Ashkenazi Jews (Streuwing et al., 1997, *New Engl. J. Med.* 336:1401). One rare tumor-associated mutant BRCA1 gene (5677insA) (Friedman et al., 1994, *Nat. Genet.* 8:399) retained ER-α repressor activity. Retention of this activity may explain the rarity of this mutation in breast cancer families. Additionally, associated genetic factors may also contribute to the high breast cancer incidence in BRCA1 kindreds (Streuwing et al., 1997, *New Engl. J. Med.* 336:1401). Furthermore, the frequency of ER-α negativity (Johannsson et al., 1997, *Eur. J. Cancer* 33:362; Loman et al., 1998, *Cancer* 83:310) and of chromosomal deletions (Tirkkonen et al., 1997, *Cancer Res.* 57:1222) are higher in BRCA1 mutant than sporadic breast cancers. Thus, the accumulation of other genetic alterations may allow estrogen-independent growth despite a BRCA1 mutation that retains the ability to repress ER-α.

Both *in vivo* and *in vitro* studies have linked expression of BRCA1 with differentiation of specific cell types such as mammary epithelium; but molecular targets of BRCA1 for mammary differentiation have not been identified (Marquis et al., 1995, *Nat. Genet.* 11:17; Rajan et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93:3078; Rajan et al., 1997, *Devel. Biol.* 184:385). The discovery of the present invention indicates that BRCA1 may function, in part, to suppress estrogen-dependent mammary epithelial proliferation by inhibiting ER-α mediated transcriptional pathways related to cell proliferation. Therefore, modulation of the estrogen signalling pathway or the estrogen receptor activation by the methods of the invention is useful in the treatment and prevention of proliferative disease (such as breast cancer). A model for this putative tumor suppressor function of BRCA1 is shown in Fig. 7. Two noteworthy features of this model are that: 1) induction of BRCA1 expression that occurs when resting cells re-enter the cycle (Gudas et al., 1995, *Cancer Res.* 55:4561; Marks et al., 1997, *Oncogene* 14:115) serves as a feedback mechanism to limit

estrogen stimulation of proliferation; and 2) BRCA1 is also postulated to promote mammary cell differentiation, by an, as yet, unidentified transcriptional pathway(s). The present invention serves to illustrate the transcriptional pathway (e.g., via the ER-α activation) which plays a role in proliferation and differentiation.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

WHAT IS CLAIMED:

1. A method of identifying modulators of the estrogen signalling pathway comprising;

(a) contacting a cell expressing an estrogen receptor and BRCA1 with an estrogen receptor ligand and a test compound;

(b) measuring the amount of proliferation exhibited by the cell;

(c) comparing the amount of measured proliferation of step (b) to that of a cell contacted solely with the estrogen receptor ligand wherein a difference between the amount of proliferation exhibited by the two cells indicates that the test compound is a modulator of the estrogen signalling pathway.

2. A method of identifying modulators of the estrogen signalling pathway comprising;

(a) contacting a cell expressing an estrogen receptor, BRCA1 and a reported gene whose expression is controlled by an estrogen response enhancer with an estrogen receptor ligand and a test compound;

(b) measuring the amount of transcription or translation of the reporter gene exhibited by the cell;

(c) comparing the amount of measured transcription or translation of step (b) to that of a cell contacted solely with the estrogen receptor ligand wherein a difference between the amount of transcription or translation exhibited by the two cells indicates that the test compound is a modulator of the estrogen signalling pathway.

3. The method of Claim 2. wherein the reporter gene is a luciferase gene. a chloramphenicol acetyltransferase gene. a human growth hormone gene. a β-galactosidase gene or a green fluorescent protein gene.

4. A method of identifying individuals at risk of developing cancer by examining the nucleotide sequence their BRCA1 domains essential for function. wherein the domains are any one in the group comprising the N-terminal RING finger domain. the consensus retinoblastoma binding motif and a domain in the C-terminal 550 amino acids.

5. The method of Claim 4 wherein the cancer is breast, ovarian, or prostate cancer.

6. A method of preventing or treating a proliferative disease in individuals identified in accordance with Claim 4, comprising administering an effective amount of a compound capable of modulating or inhibiting an estrogen signalling pathway in a host such that cell proliferation is decreased or inhibited.

7. The method of Claim 6, wherein the proliferative disease is cancer.

8. The method of Claim 6, wherein the cancer affects breast ovarian or prostate tissue.

9. The method of Claim 6 wherein the compound inhibits transcriptional activation through estrogen response enhancers.

10. The method of Claim 9 where in the compound inhibits binding of at least one component of the estrogen coactivating complex to estrogen response enhancers.

11. The method of Claim 10 wherein the component of the estrogen coactivating complex is p300 or CBP

12. A method for decreasing or inhibiting tumor growth in a mammal comprising administering to said mammal an amount of a compound that inhibits signalling by the estrogen receptor through estrogen responsive enhancers effective to decrease or inhibit tumor growth in said mammal.

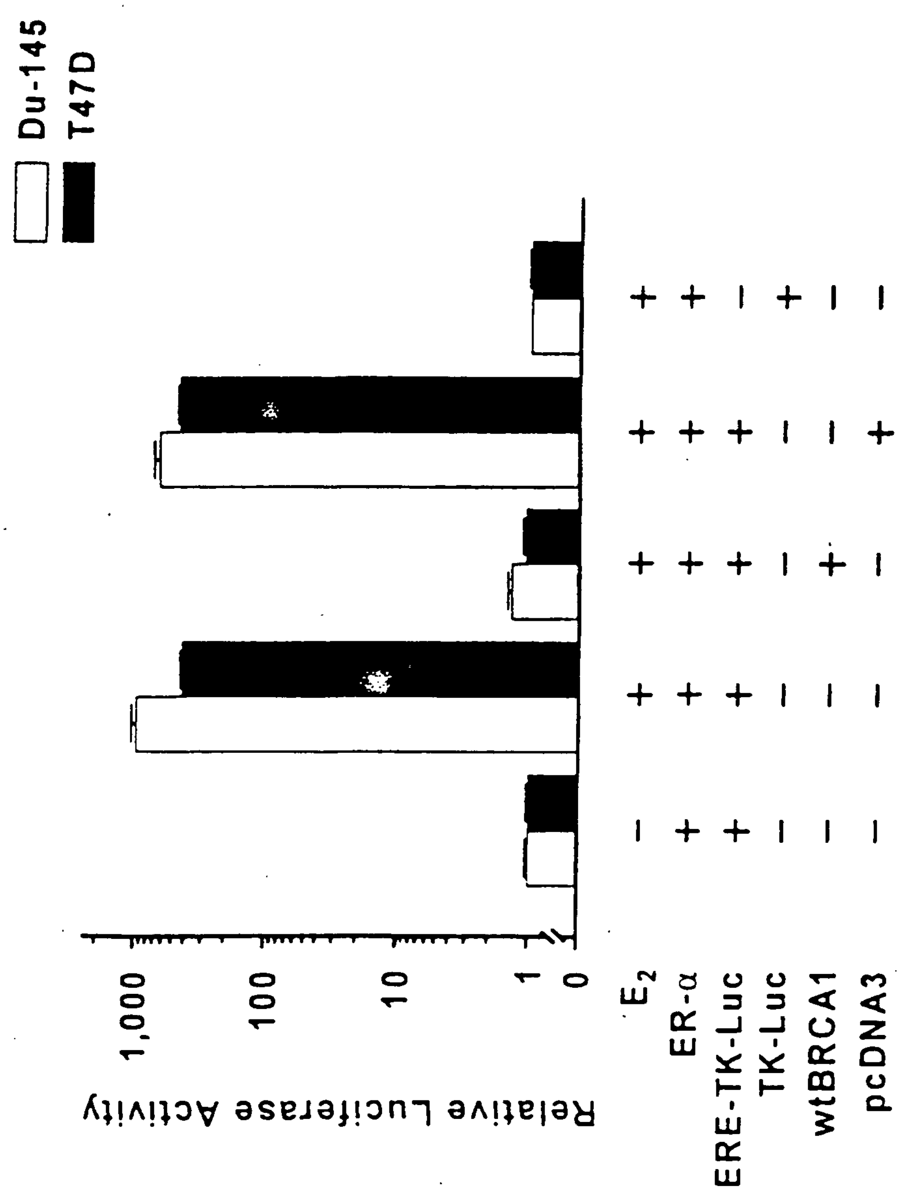

Du-145
T47D
Relative Luciferase Activity
1,000
100
10
1
0
E_2
ER-α
ERE-TK-Luc
TK-Luc
wtBRCA1
pcDNA3

FIG.1A

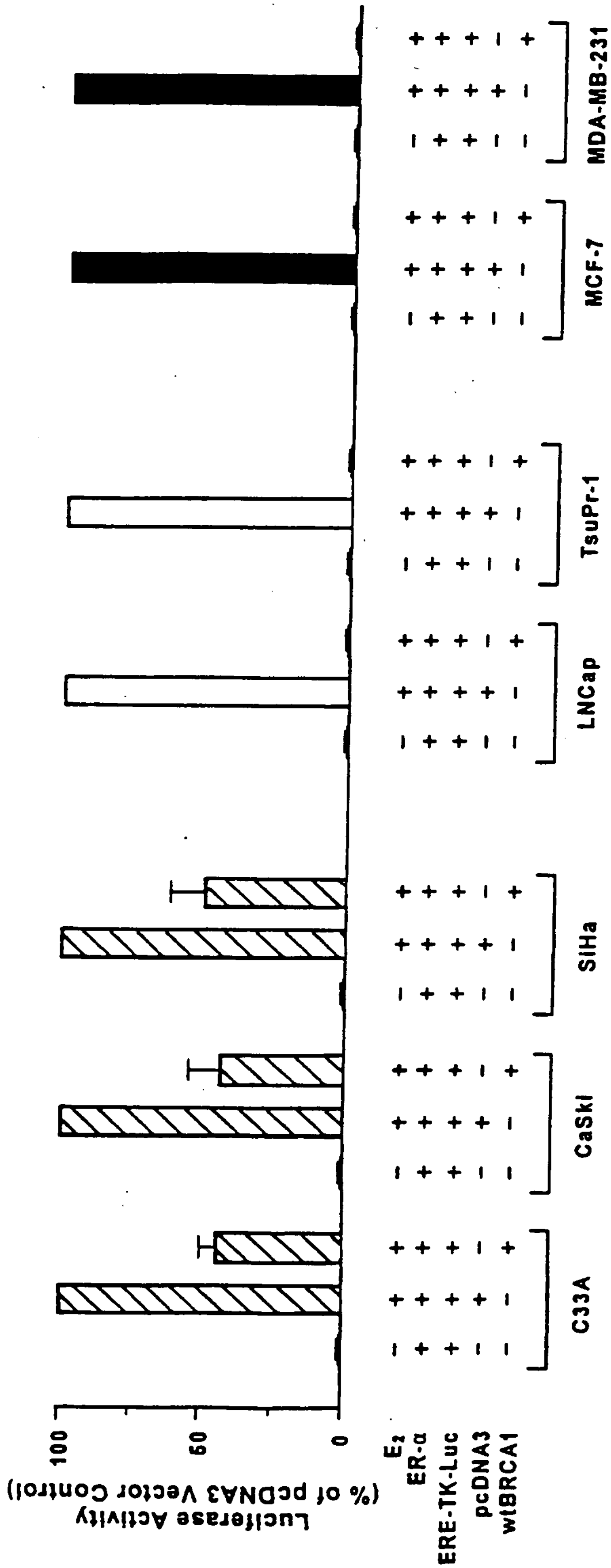

Luciferase Activity
(% of pcDNA3 Vector Control)
100
50
0
E2
ER-α
ERE-TK-Luc
pcDNA3
wtBRCA1
C33A
CaSki
SiHa
LNCap
TsuPr-1
MCF-7
MDA-MB-231

FIG1B

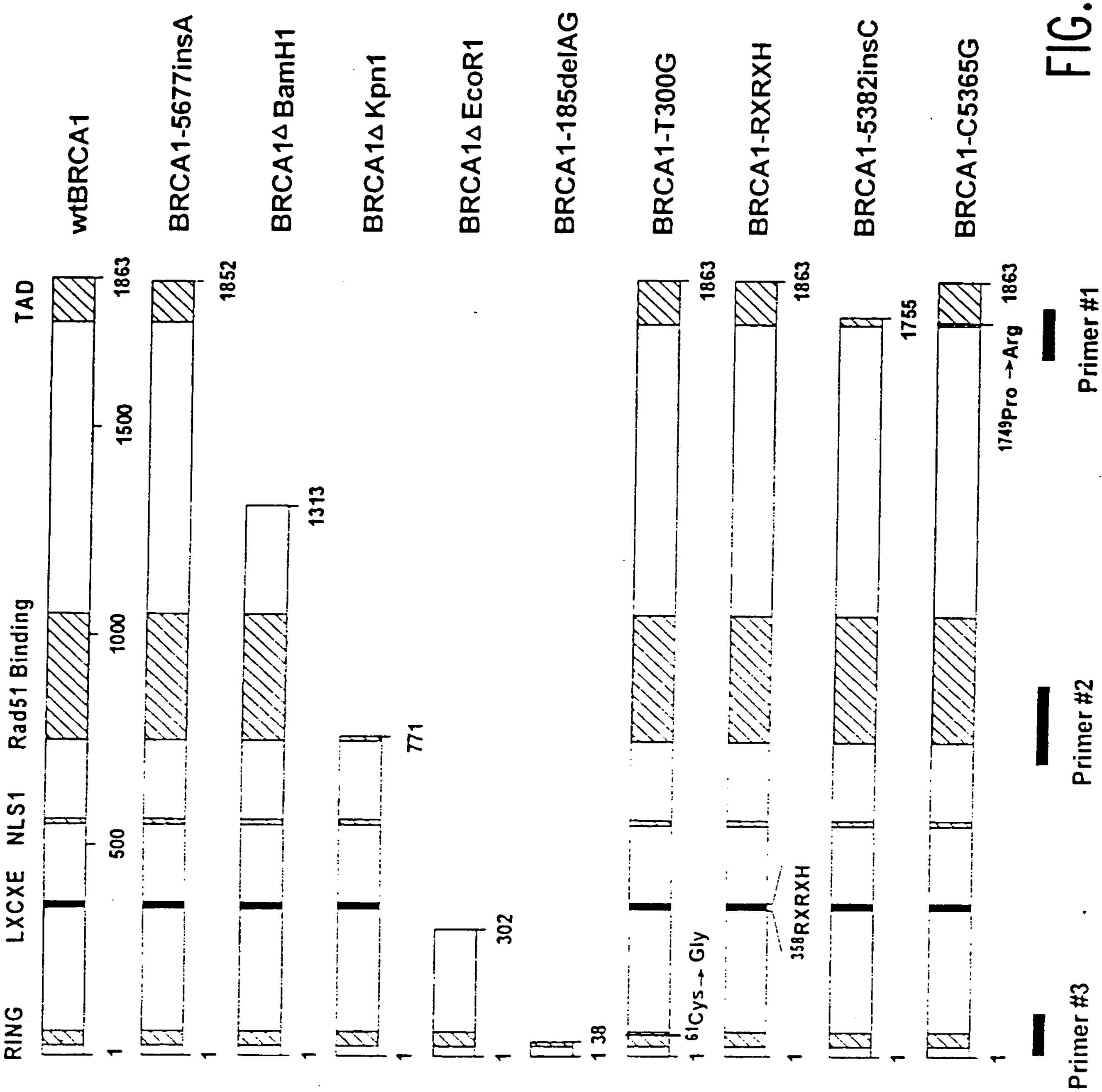

RING
LXCXE
NLS1
Rad51 Binding
TAD
1
500
1000
1500
1863
wtBRCA1
BRCA1-5677insA
1852
BRCA1Δ BamH1
1313
BRCA1Δ Kpn1
771
BRCA1Δ EcoR1
302
BRCA1-185delAG
1 38
BRCA1-T300G
61Cys→ Gly
BRCA1-RXRXH
358RXRXH
BRCA1-5382insC
1755
BRCA1-C5365G
1749Pro →Arg
Primer #1
Primer #2
Primer #3

FIG.1C

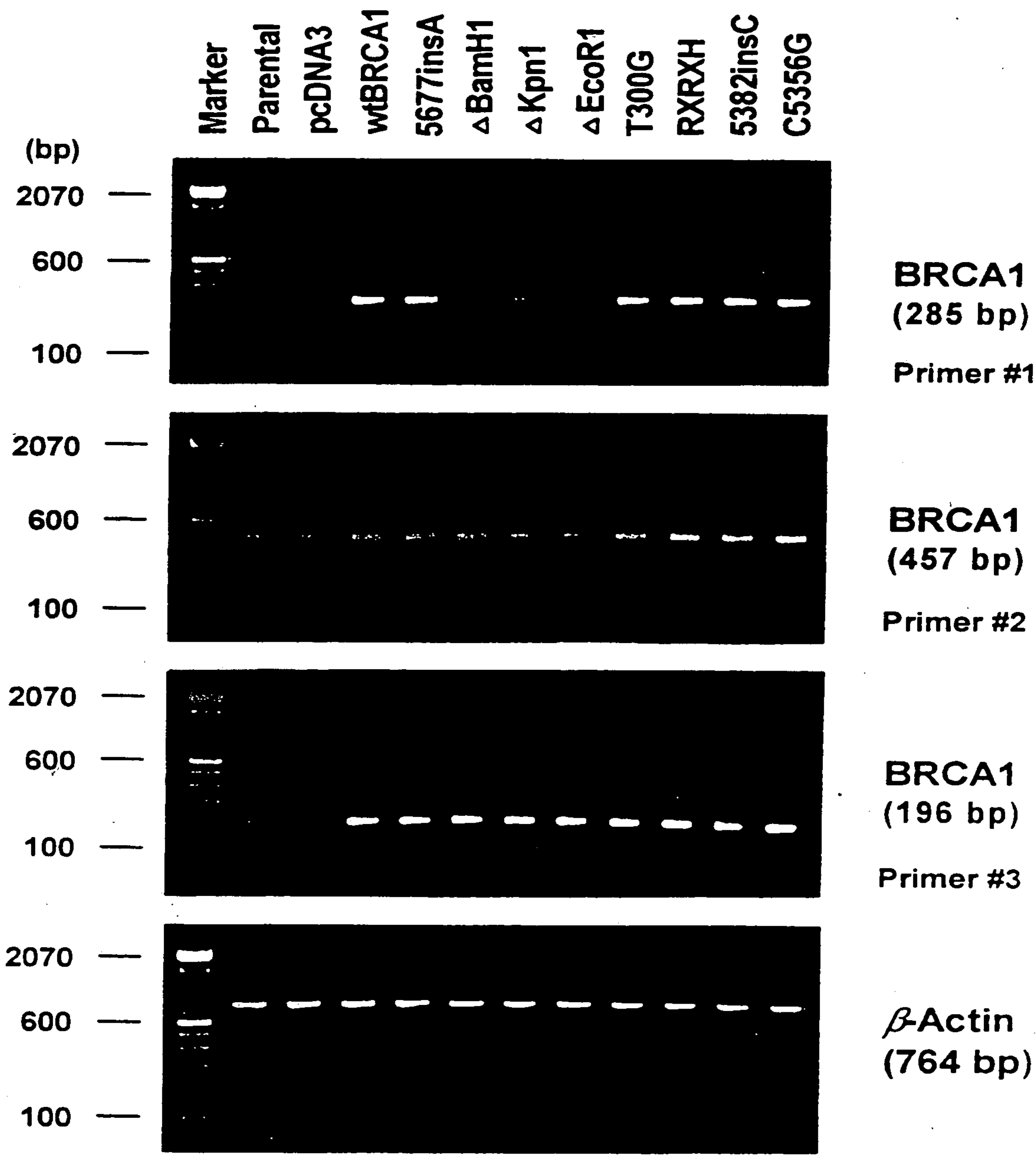

Marker
Parental
pcDNA3
wtBRCA1
5677insA
ΔBamH1
ΔKpn1
ΔEcoR1
T300G
RXRXH
5382insC
C5356G
(bp)
2070
600
100
BRCA1
(285 bp)
Primer #1
2070
600
100
BRCA1
(457 bp)
Primer #2
2070
600
100
BRCA1
(196 bp)
Primer #3
2070
600
100
β-Actin
(764 bp)

FIG.1D

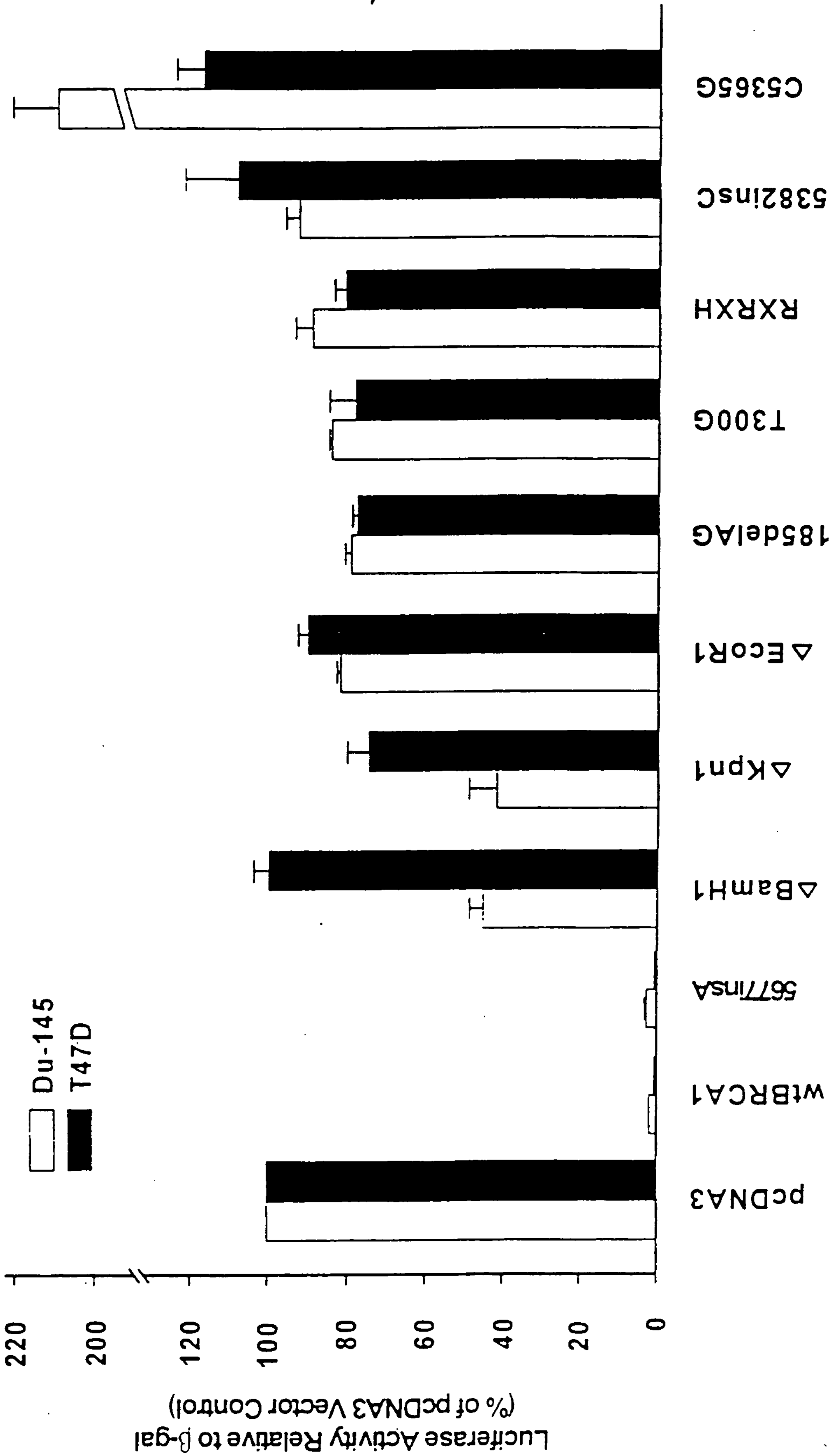

Du-145
T47D
C5365G
5382insC
RXRXH
T300G
185delAG
ΔEcoR1
ΔKpn1
ΔBamH1
5677insA
wtBRCA1
pcDNA3
220
200
120
100
80
60
40
20
0
Luciferase Activity Relative to β-gal
(% of pcDNA3 Vector Control)

FIG.1E

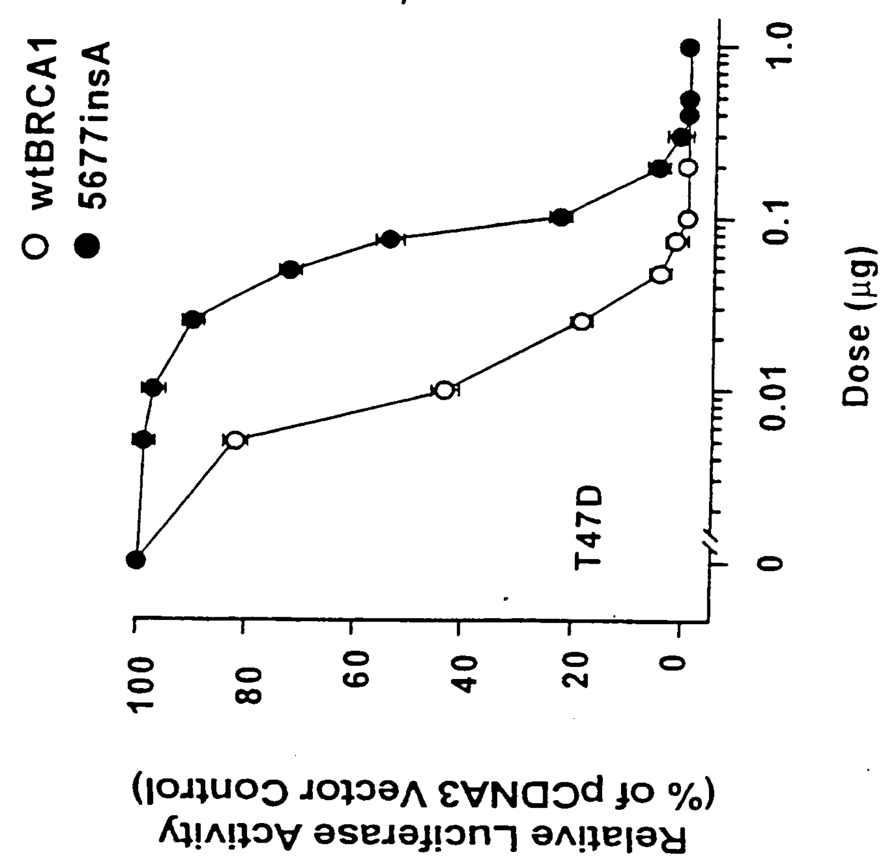


FIG.1F-B

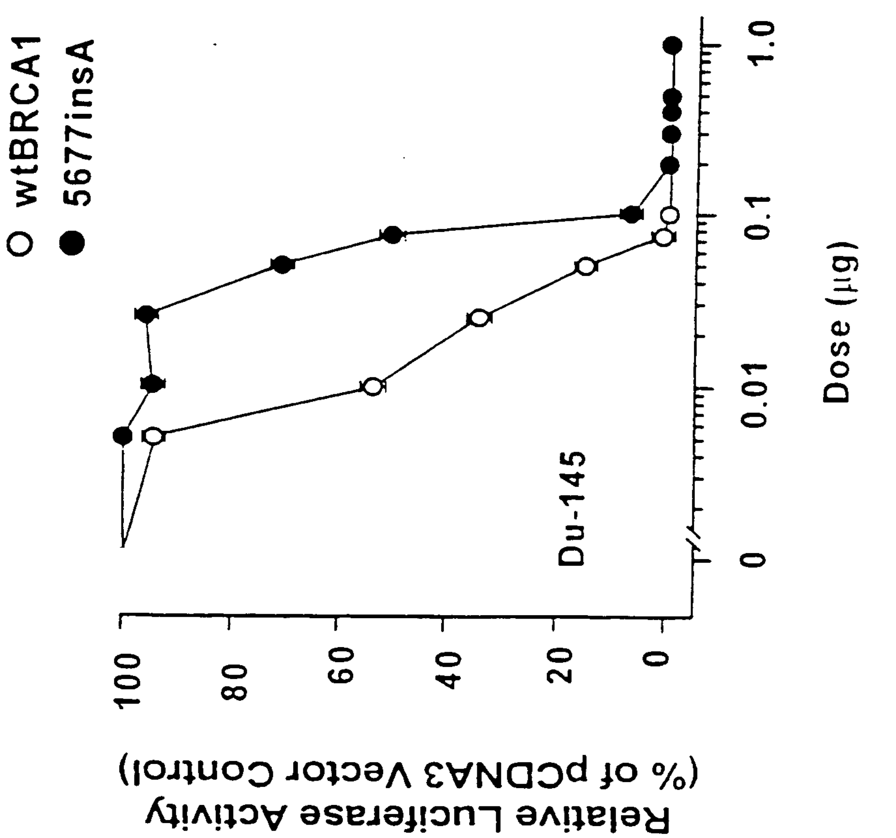
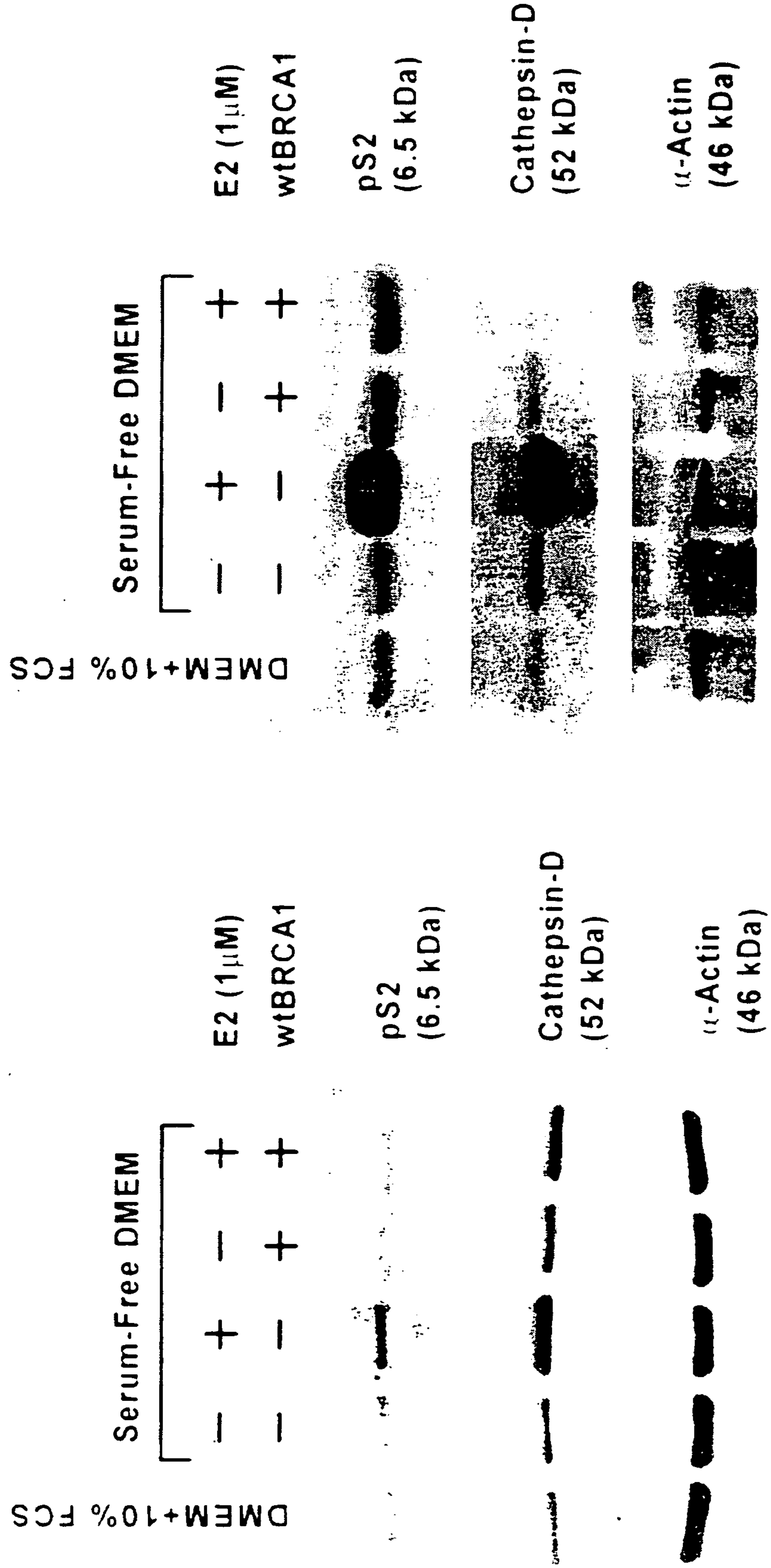


FIG.1F-A



Serum-Free DMEM
DMEM+10% FCS
E2 (1μM)
wtBRCA1
pS2
(6.5 kDa)
Cathepsin-D
(52 kDa)
α-Actin
(46 kDa)
Serum-Free DMEM
DMEM+10% FCS
E2 (1μM)
wtBRCA1
pS2
(6.5 kDa)
Cathepsin-D
(52 kDa)
α-Actin
(46 kDa)

FIG.1G

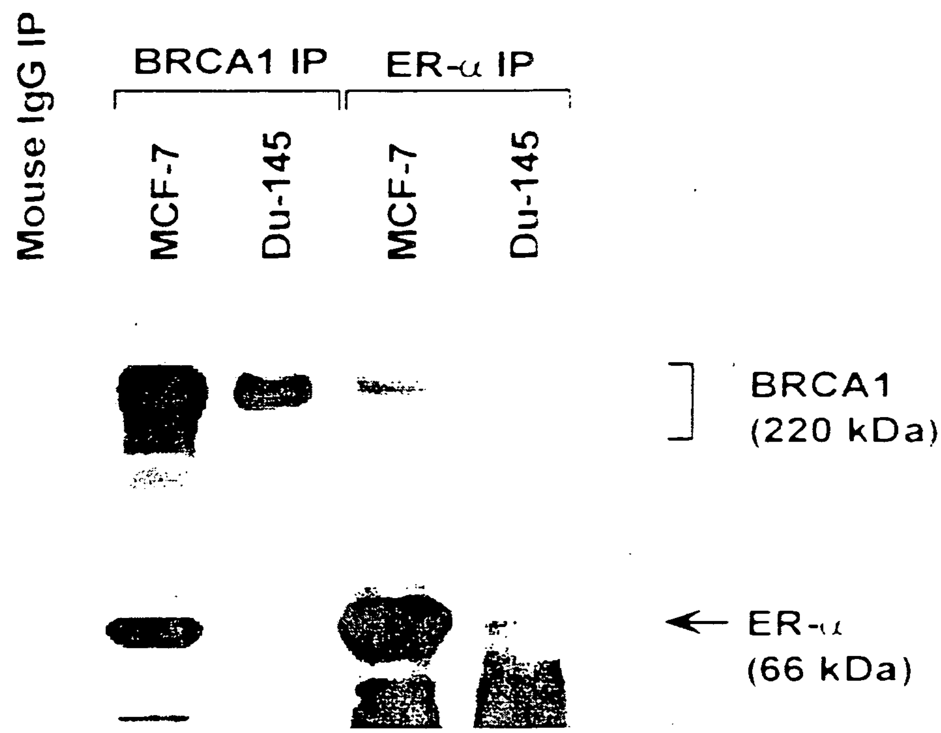

Mouse IgG IP
BRCA1 IP
ER-α IP
MCF-7
Du-145
MCF-7
Du-145
BRCA1
(220 kDa)
ER-α
(66 kDa)

FIG.2A

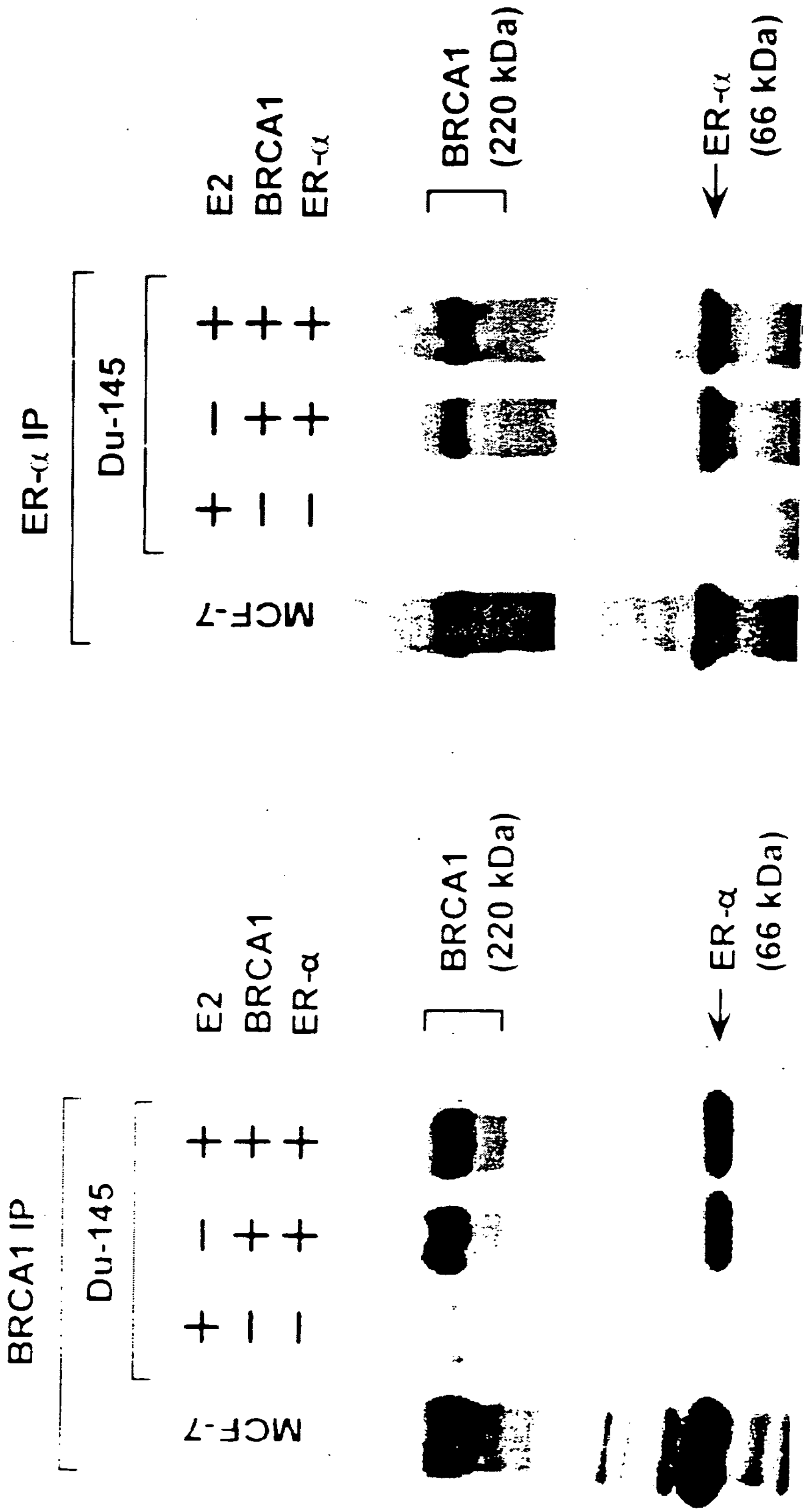

ER-α IP
Du-145
MCF-7
E2
BRCA1
ER-α
BRCA1
(220 kDa)
ER-α
(66 kDa)
BRCA1 IP
Du-145
MCF-7
E2
BRCA1
ER-α
BRCA1
(220 kDa)
ER-α
(66 kDa)

FIG.2B

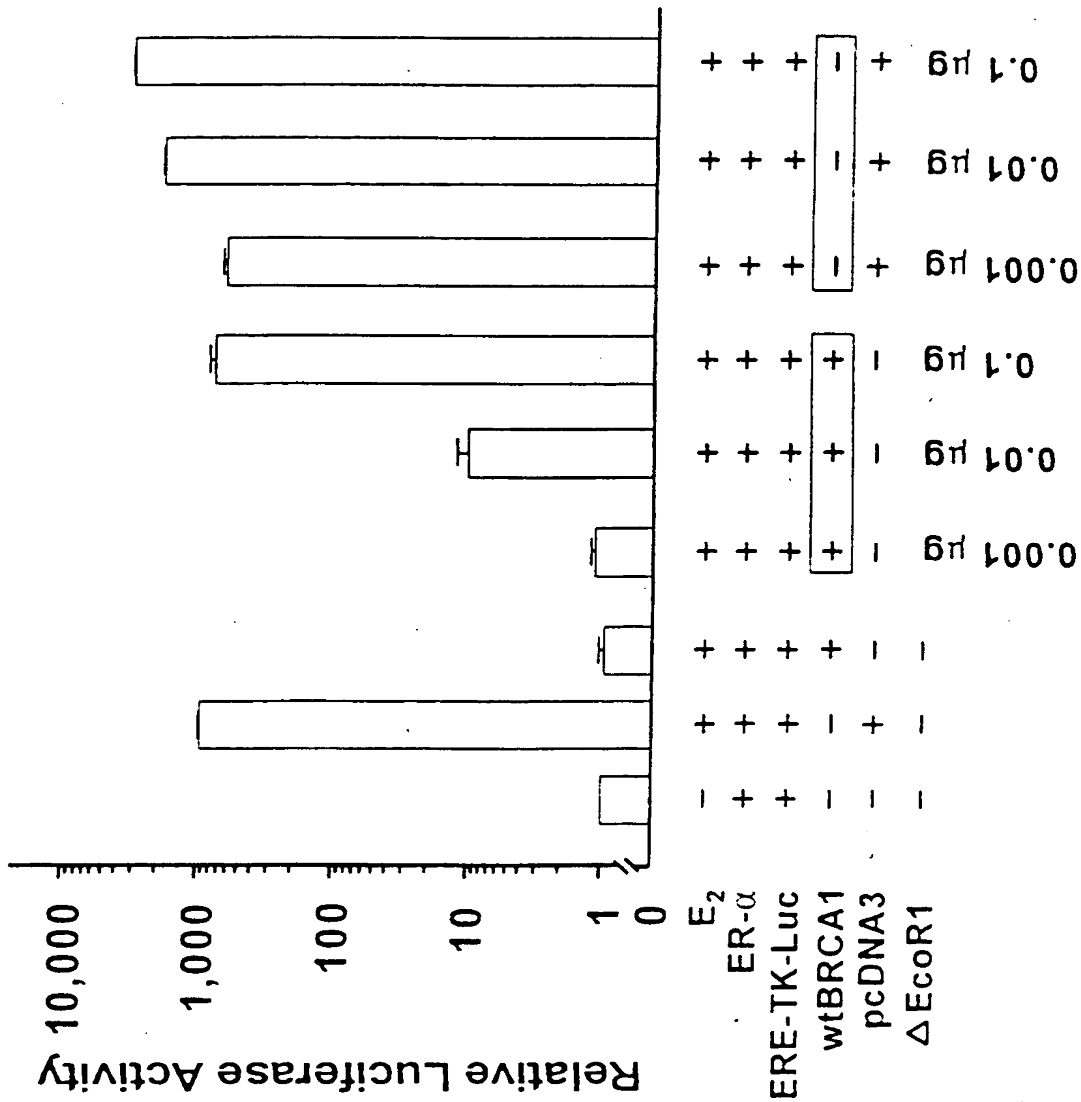
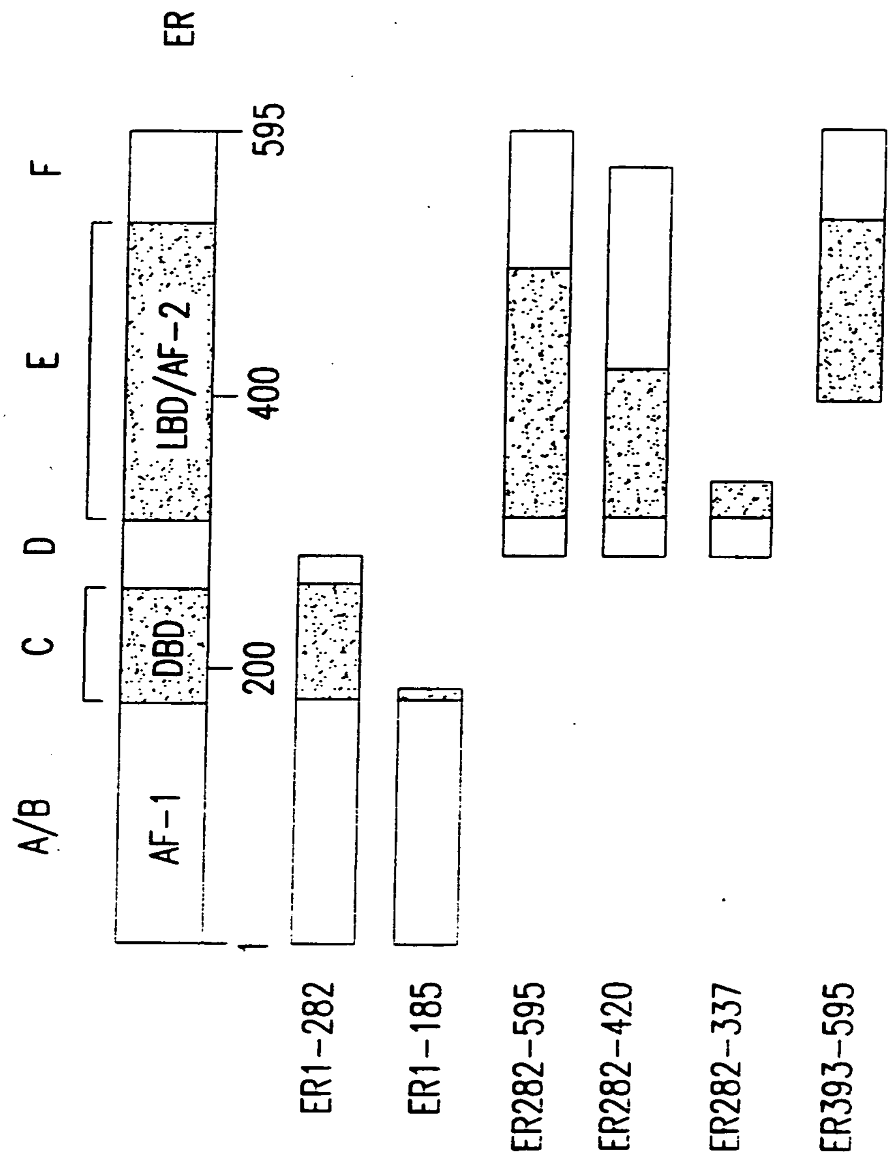

Relative Luciferase Activity
10,000
1,000
100
10
1
0
E$_2$
ER-α
ERE-TK-Luc
wtBRCA1
pcDNA3
ΔEcoR1
0.001 μg
0.01 μg
0.1 μg
0.001 μg
0.01 μg
0.1 μg

FIG.2C



A/B
C
D
E
F
AF-1
DBD
LBD/AF-2
ER
1
200
400
595
ER1-282
ER1-185
ER282-595
ER282-420
ER282-337
ER393-595

FIG.3A

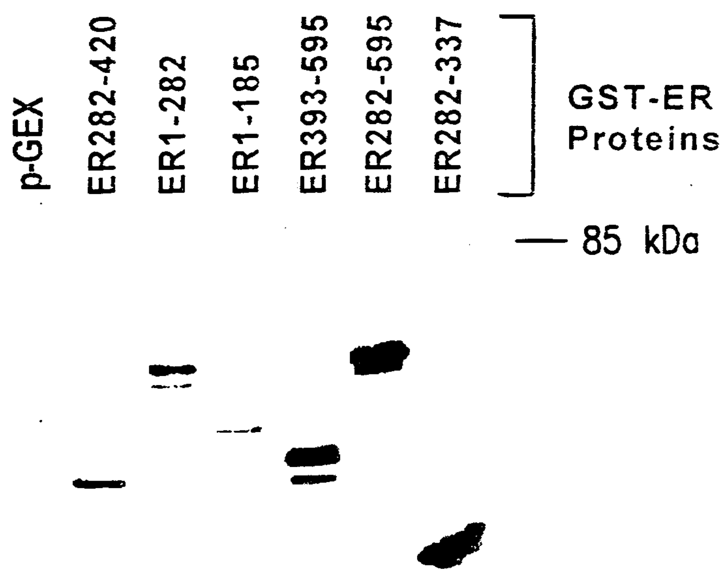

p-GEX
ER282-420
ER1-282
ER1-185
ER393-595
ER282-595
ER282-337
GST-ER Proteins
85 kDa

FIG.3B

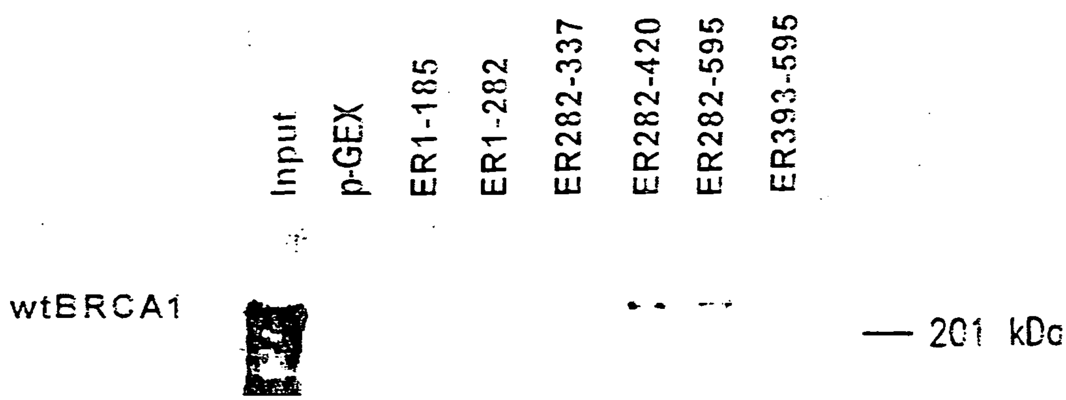

Input
p-GEX
ER1-185
ER1-282
ER282-337
ER282-420
ER282-595
ER393-595
wtBRCA1
201 kDa

FIG.3C

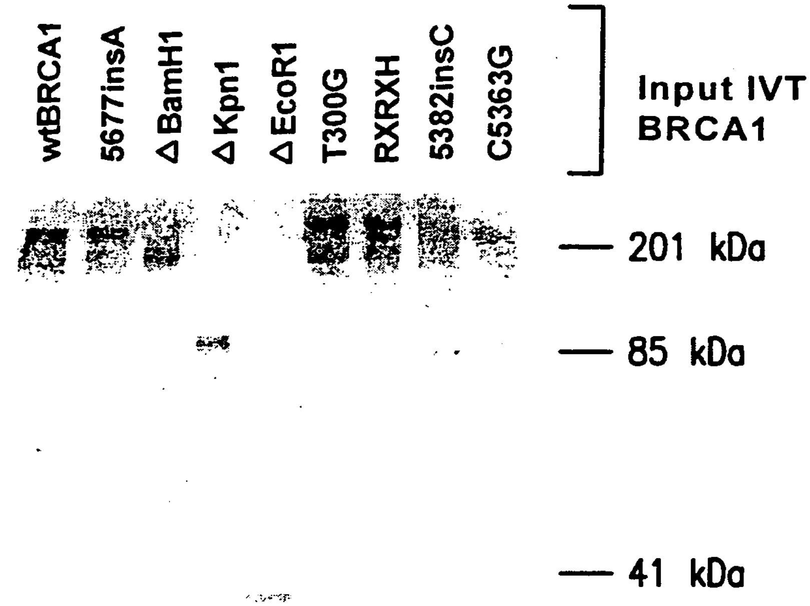

wtBRCA1
5677insA
Δ BamH1
Δ Kpn1
Δ EcoR1
T300G
RXRXH
5382insC
C5363G
Input IVT BRCA1
201 kDa
85 kDa
41 kDa

FIG.3D

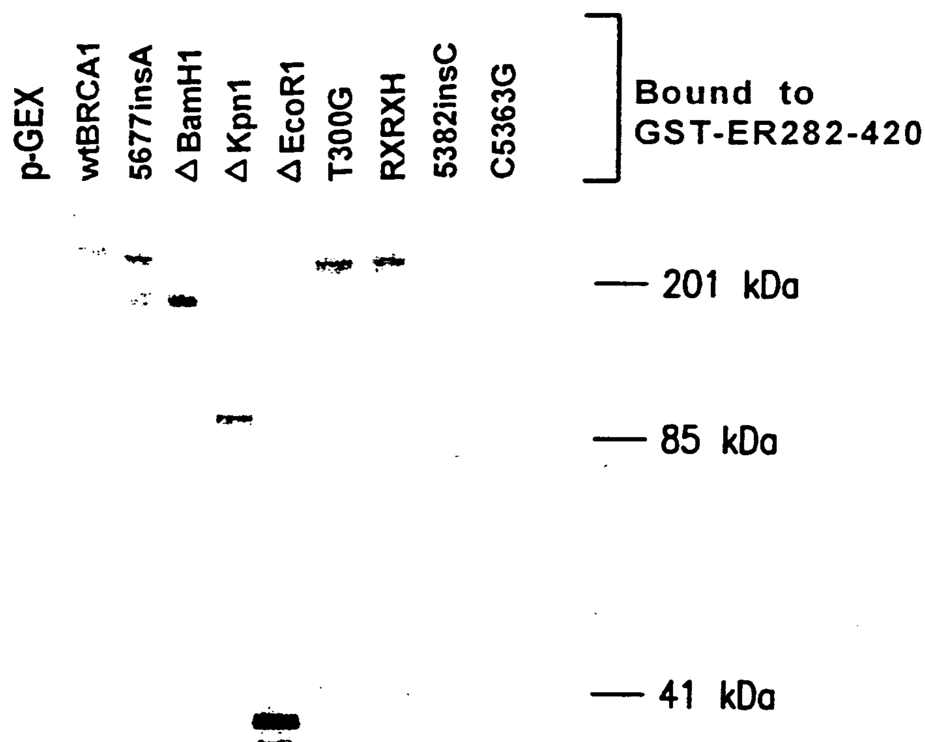

p-GEX
wtBRCA1
5677insA
Δ BamH1
Δ Kpn1
Δ EcoR1
T300G
RXRXH
5382insC
C5363G
Bound to GST-ER282-420
201 kDa
85 kDa
41 kDa

FIG.3E

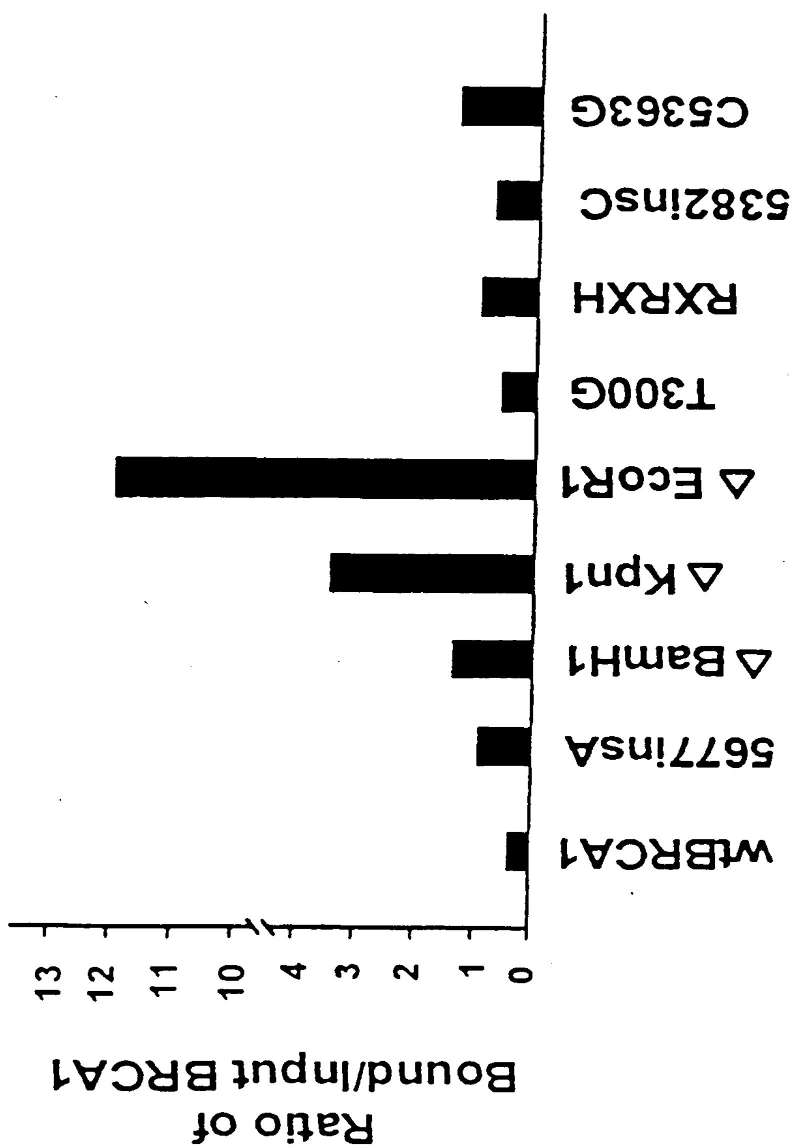

C5363G
5382insC
RXRXH
T300G
Δ EcoR1
Δ Kpn1
Δ BamH1
5677insA
wtBRCA1
0
1
2
3
4
10
11
12
13
Ratio of
Bound/Input BRCA1

FIG.3F

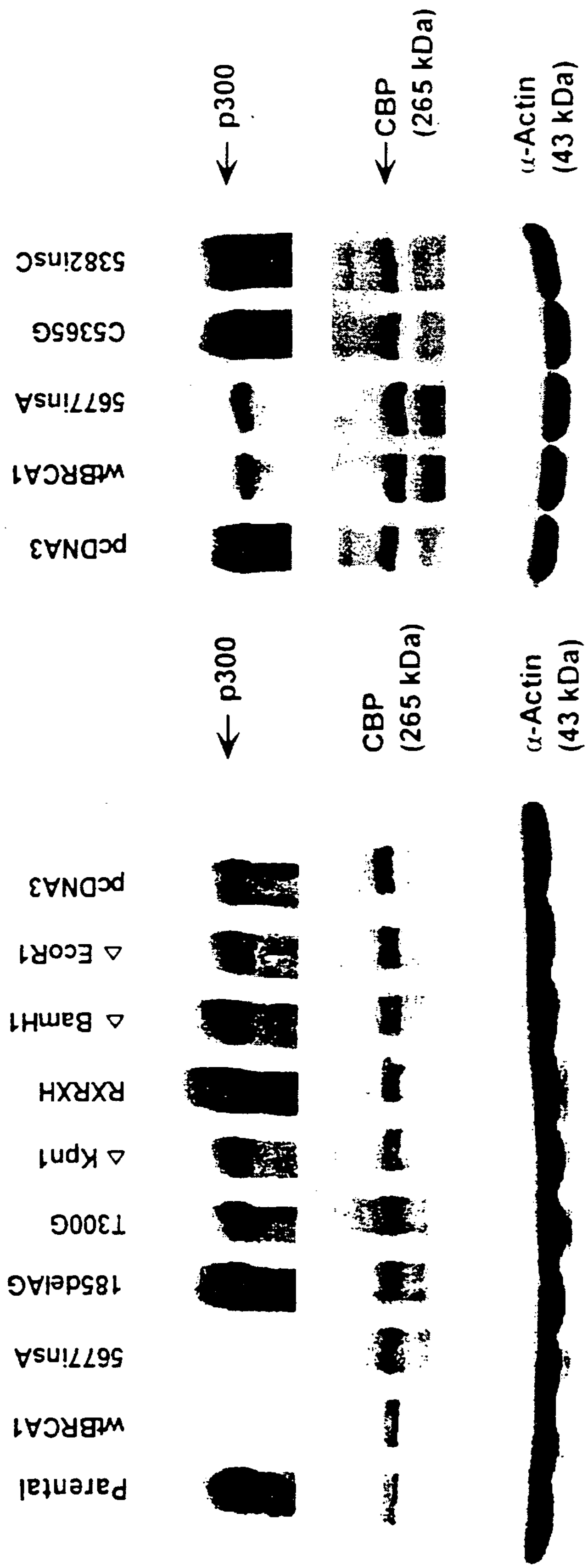

5382insC
C5365G
5677insA
wtBRCA1
pcDNA3
p300
CBP (265 kDa)
α-Actin (43 kDa)
pcDNA3
Δ EcoR1
Δ BamH1
RXRXH
Δ Kpn1
T300G
185delAG
5677insA
wtBRCA1
Parental
p300
CBP (265 kDa)
α-Actin (43 kDa)

FIG.4A

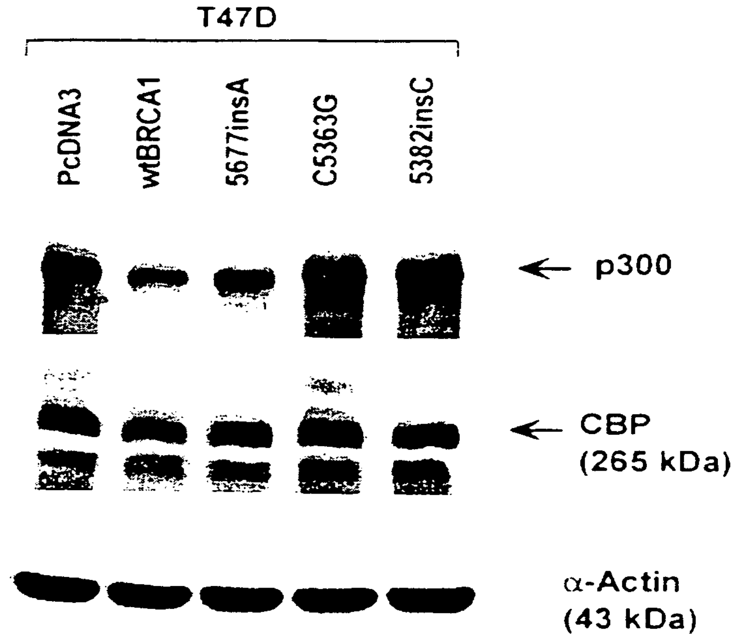

T47D
PcDNA3
wtBRCA1
5677insA
C5363G
5382insC
p300
CBP
(265 kDa)
α-Actin
(43 kDa)

FIG.4B

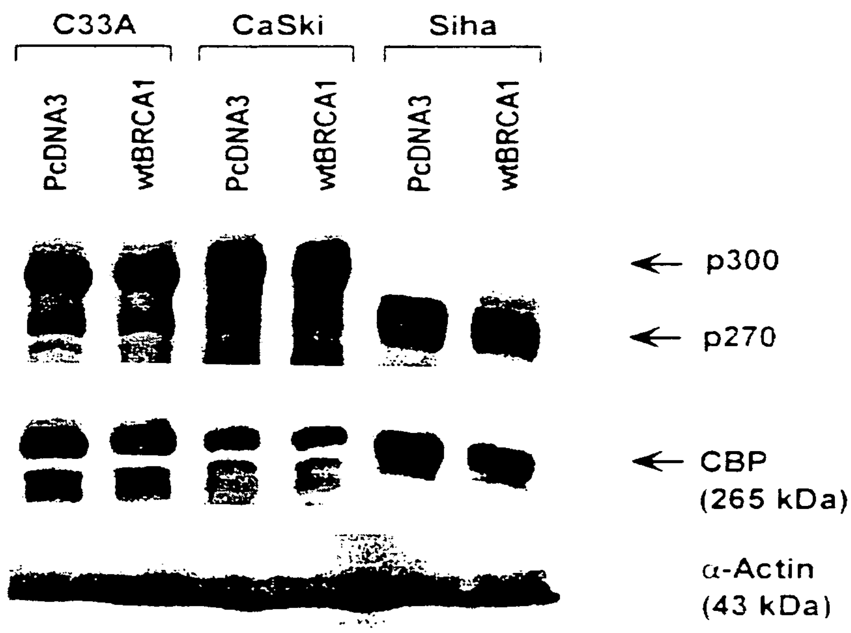

C33A
CaSki
Siha
PcDNA3
wtBRCA1
PcDNA3
wtBRCA1
PcDNA3
wtBRCA1
p300
p270
CBP
(265 kDa)
α-Actin
(43 kDa)

FIG.4C

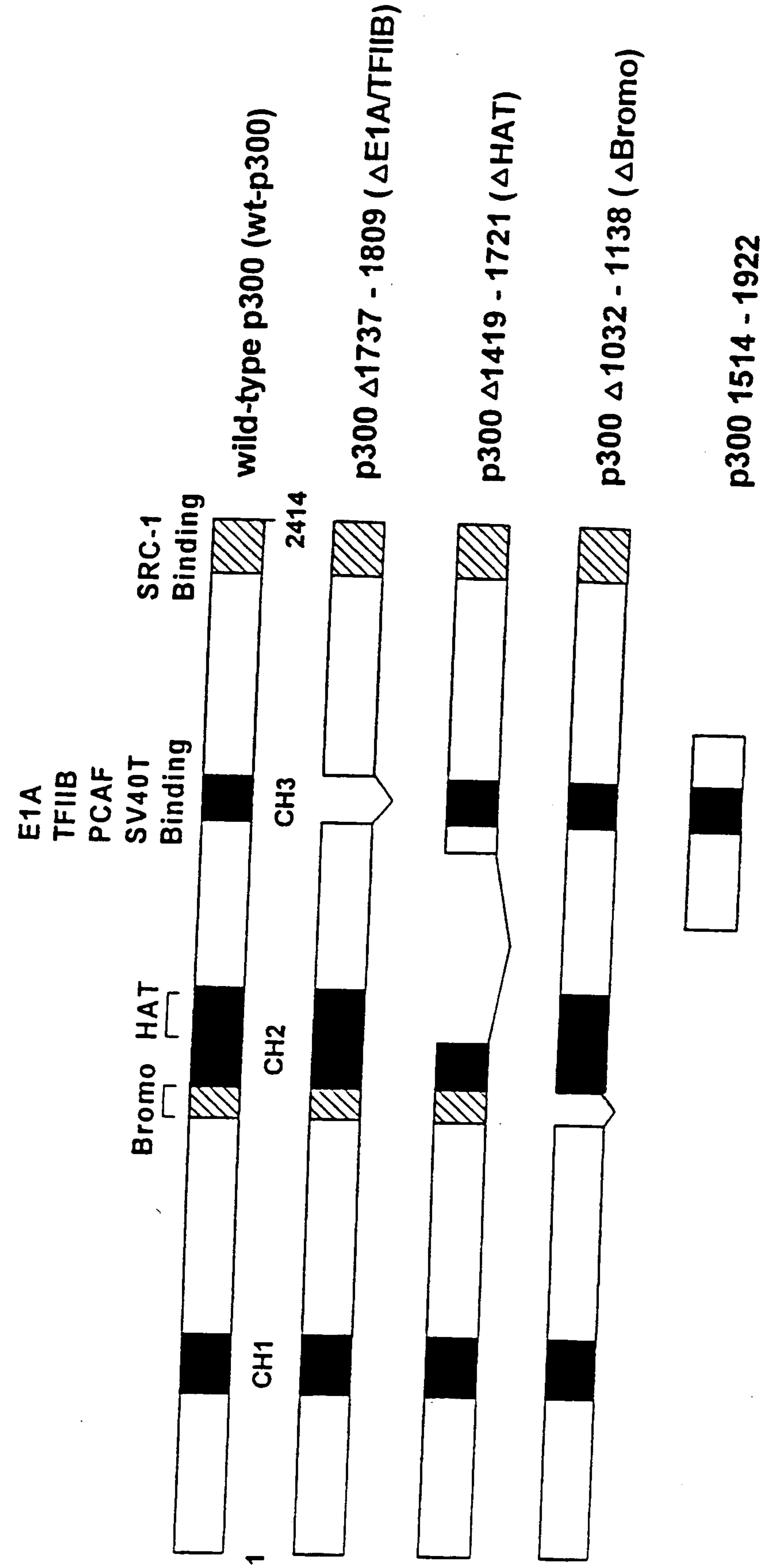

E1A
TFIIB
PCAF
SV40T
Binding
SRC-1
Binding
Bromo
HAT
CH1
CH2
CH3
1
2414
wild-type p300 (wt-p300)
p300 Δ1737 - 1809 (ΔE1A/TFIIB)
p300 Δ1419 - 1721 (ΔHAT)
p300 Δ1032 - 1138 (ΔBromo)
p300 1514 - 1922

FIG.5A

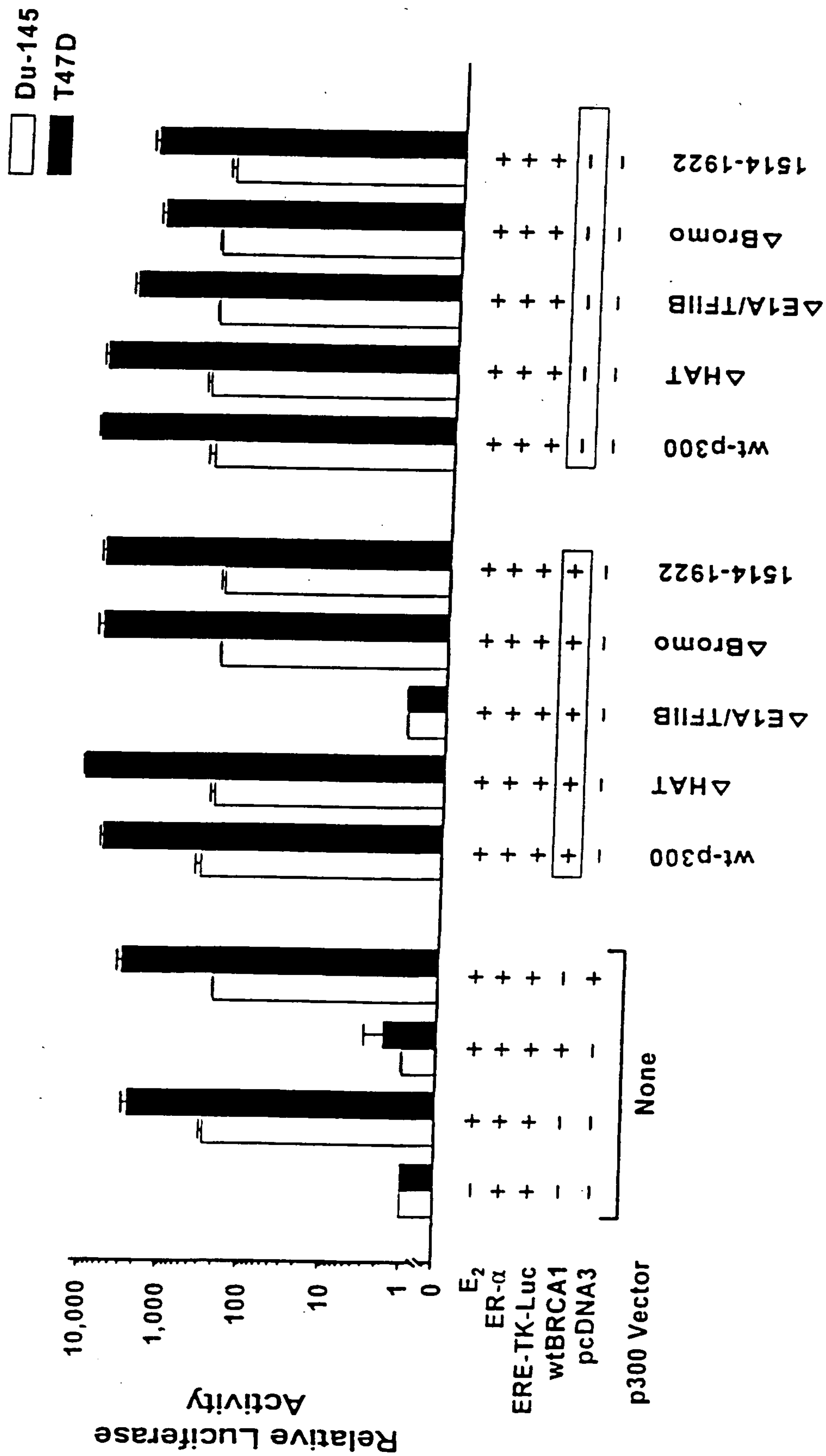

Du-145
T47D
Relative Luciferase Activity
10,000
1,000
100
10
1
0
E2
ER-α
ERE-TK-Luc
wtBRCA1
pcDNA3
p300 Vector
None
wt-p300
ΔHAT
ΔE1A/TFIIB
ΔBromo
1514-1922

FIG.5B

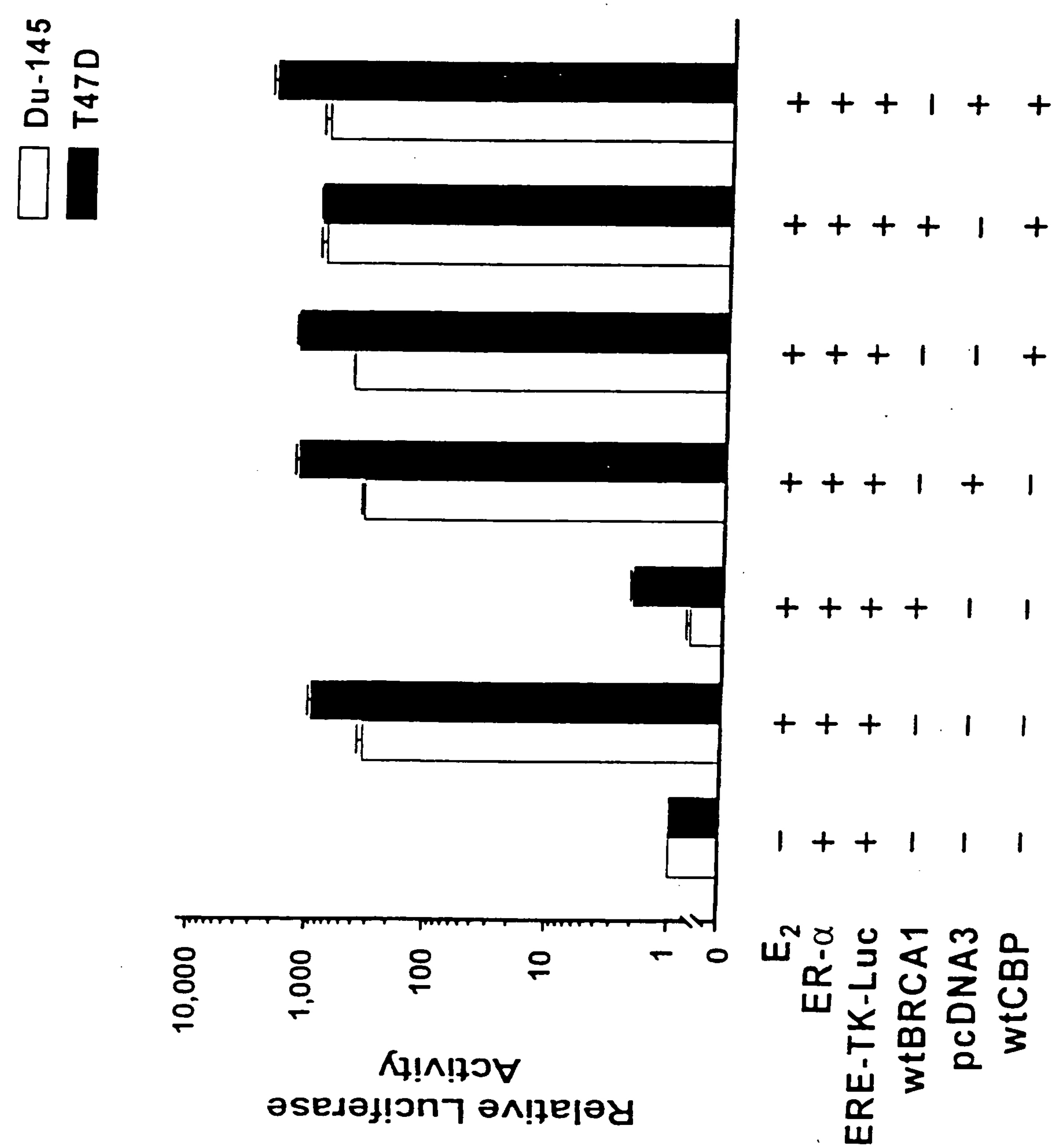

Du-145
T47D
Relative Luciferase Activity
10,000
1,000
100
10
1
0
E2
ER-α
ERE-TK-Luc
wtBRCA1
pcDNA3
wtCBP
- + + - - -
+ + + - - -
+ + + + - -
+ + + - + -
+ + + - - +
+ + + + - +
+ + + - + +

FIG.5C

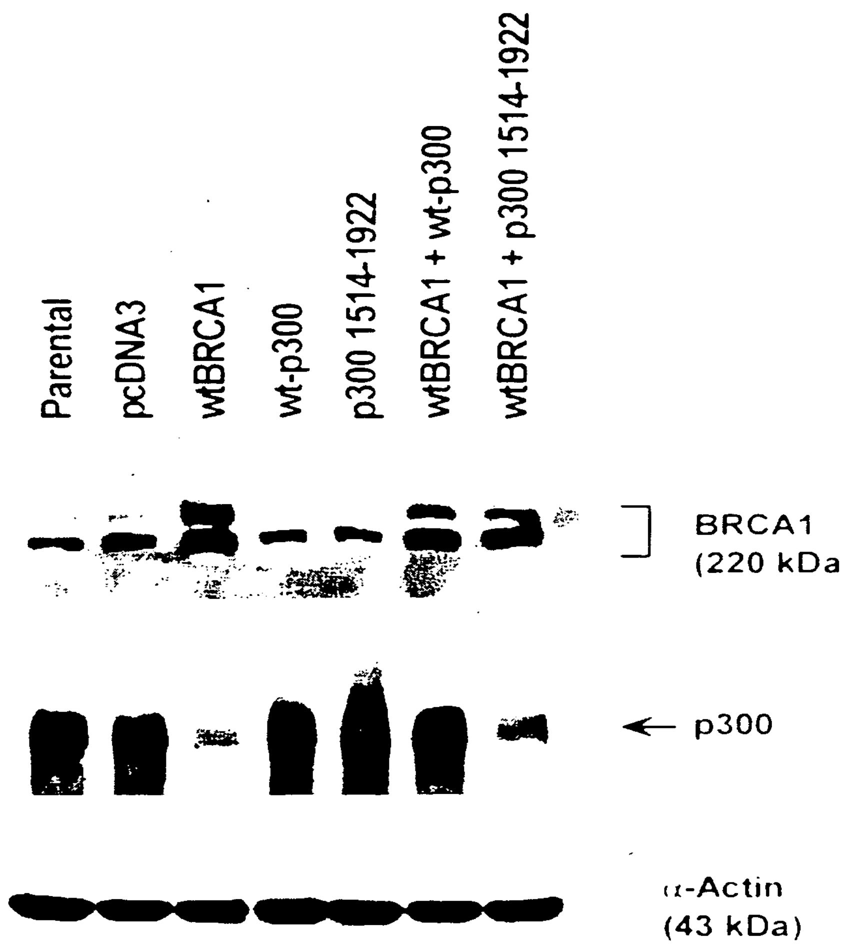

Parental
pcDNA3
wtBRCA1
wt-p300
p300 1514-1922
wtBRCA1 + wt-p300
wtBRCA1 + p300 1514-1922
BRCA1
(220 kDa)
p300
α-Actin
(43 kDa)

FIG.5D

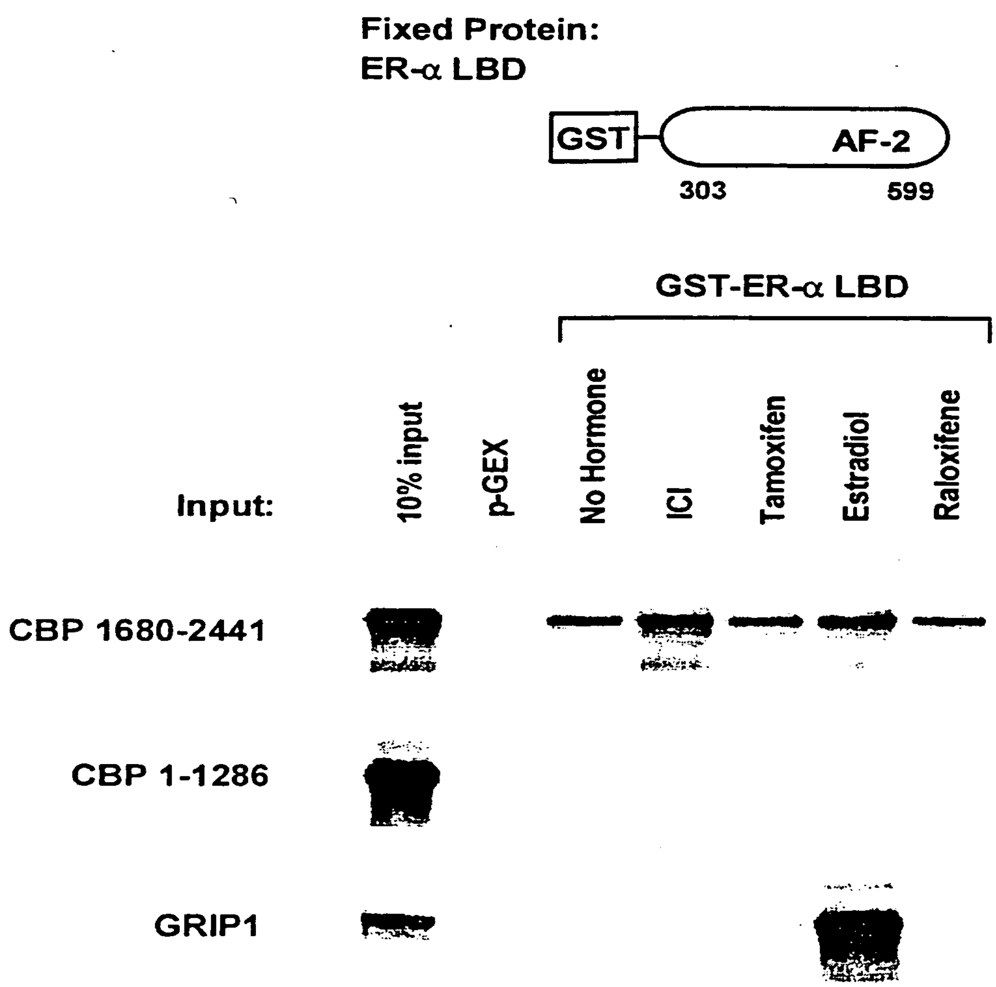

Fixed Protein:
ER-α LBD
GST
AF-2
303
599
GST-ER-α LBD
Input:
10% input
p-GEX
No Hormone
ICI
Tamoxifen
Estradiol
Raloxifene
CBP 1680-2441
CBP 1-1286
GRIP1

FIG.6A

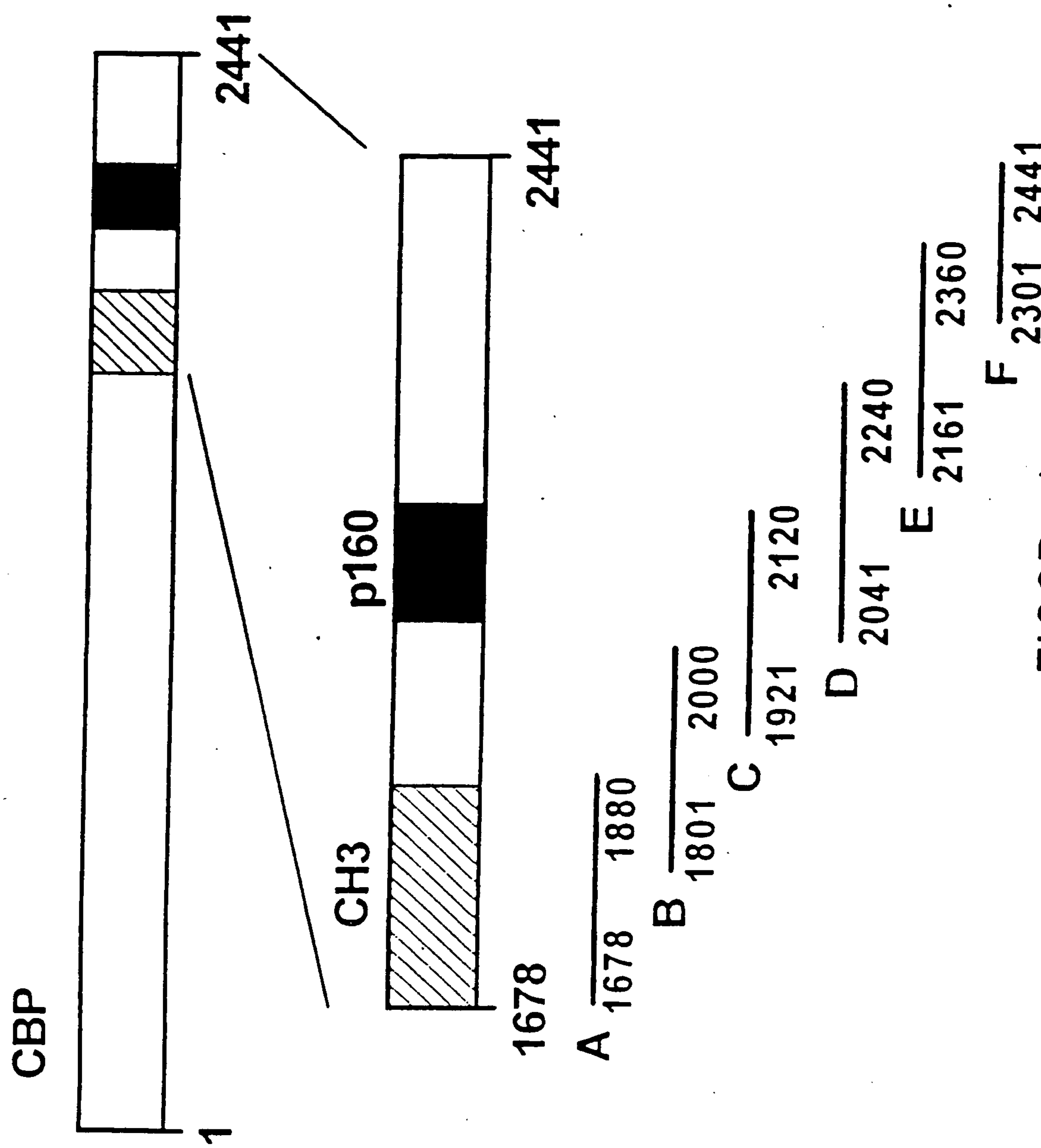

CBP
1
2441
p160
CH3
1678
2441
A 1678 1880
B 1801 2000
C 1921 2120
D 2041 2240
E 2161 2360
F 2301 2441

FIG6B-A

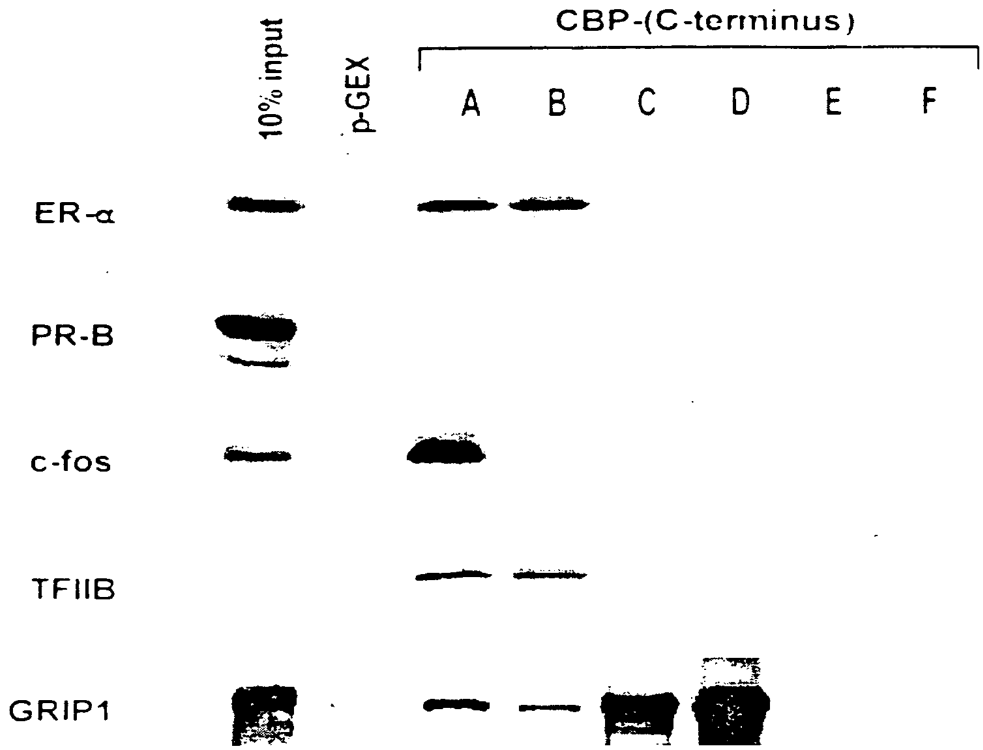


FIG.6B-B

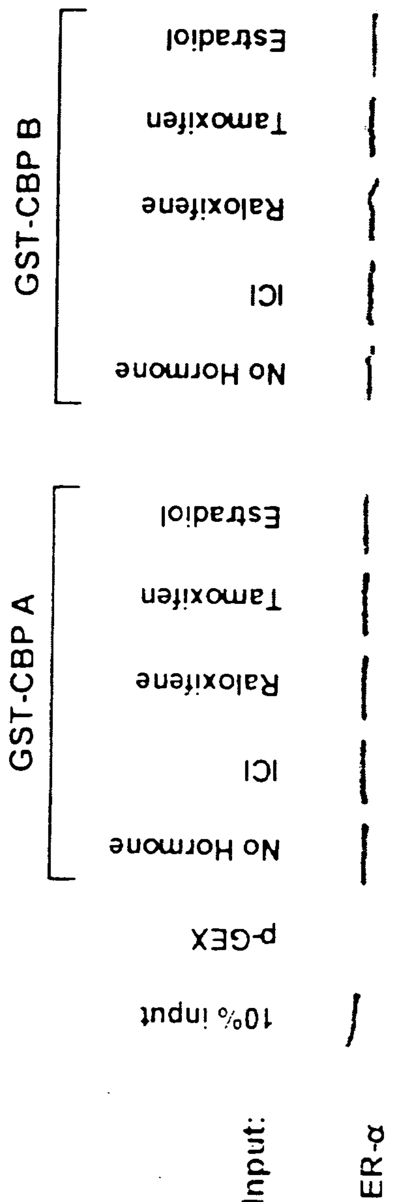

Input:
ER-α
10% input
p-GEX
GST-CBP A
No Hormone
ICI
Raloxifene
Tamoxifen
Estradiol
GST-CBP B
No Hormone
ICI
Raloxifene
Tamoxifen
Estradiol

FIG.6C

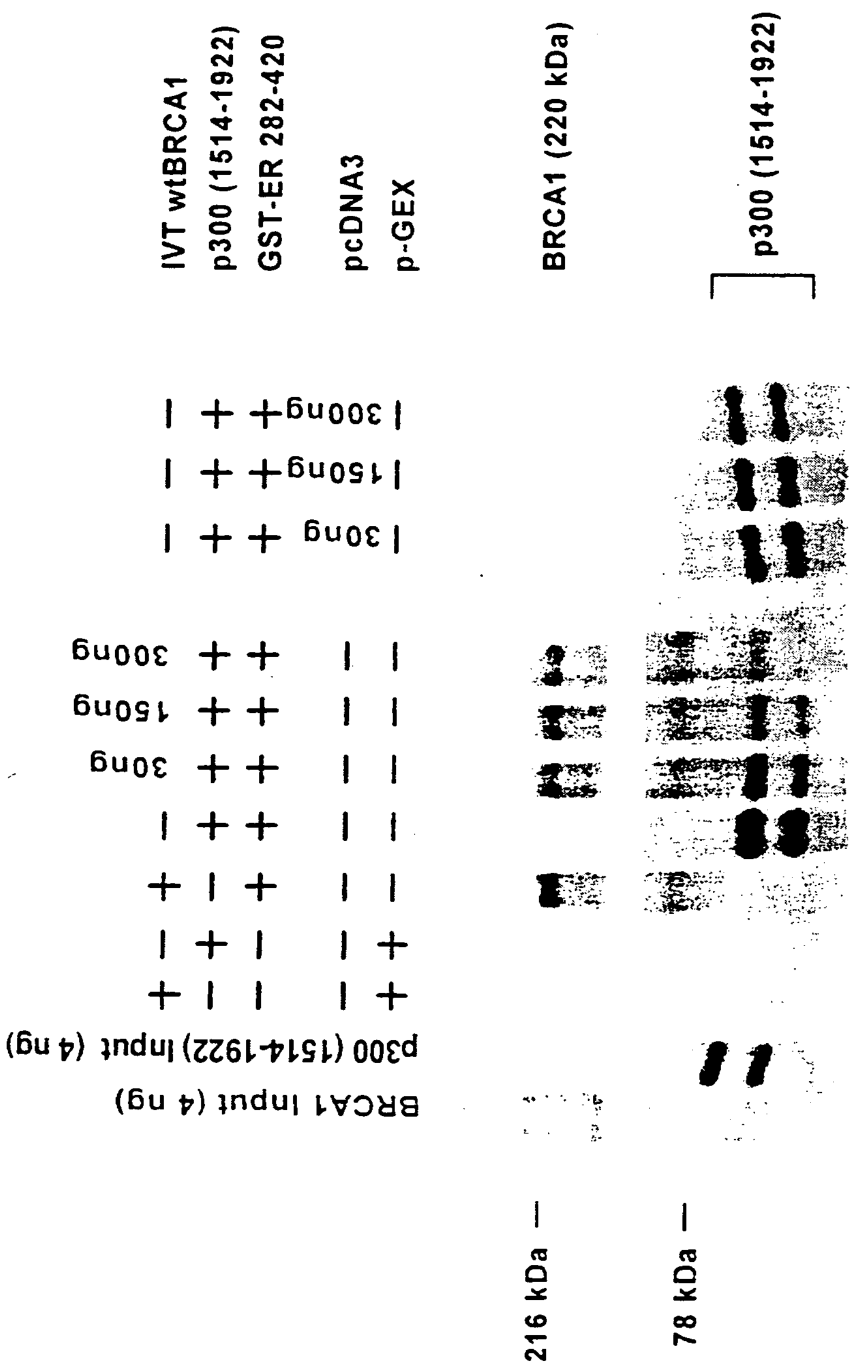

IVT wtBRCA1
p300 (1514-1922)
GST-ER 282-420
pcDNA3
p-GEX
BRCA1 (220 kDa)
p300 (1514-1922)
p300 (1514-1922) Input (4 ng)
BRCA1 Input (4 ng)
30ng
150ng
300ng
216 kDa
78 kDa

FIG.6D-A

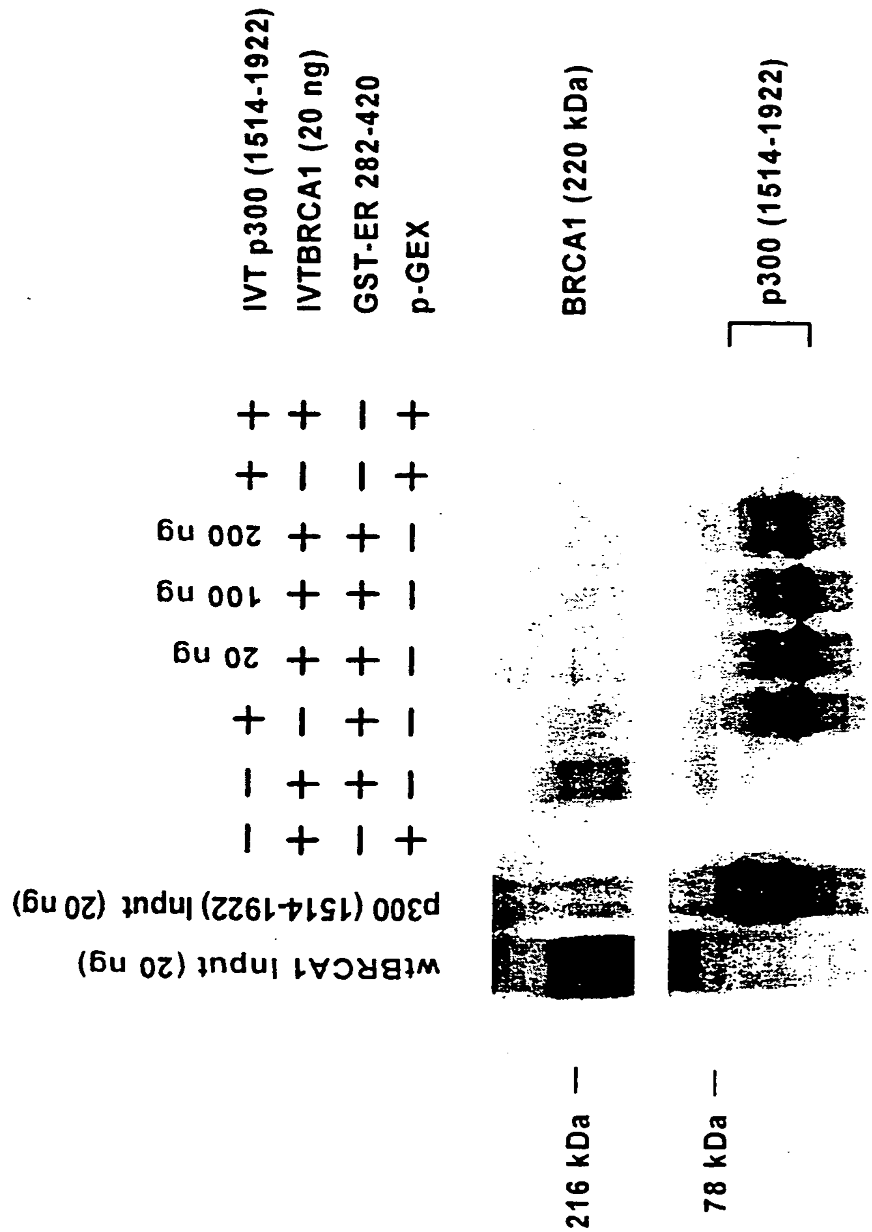

IVT p300 (1514-1922)
IVTBRCA1 (20 ng)
GST-ER 282-420
p-GEX
BRCA1 (220 kDa)
p300 (1514-1922)
200 ng
100 ng
20 ng
p300 (1514-1922) Input (20 ng)
wtBRCA1 Input (20 ng)
216 kDa
78 kDa

FIG.6D-B

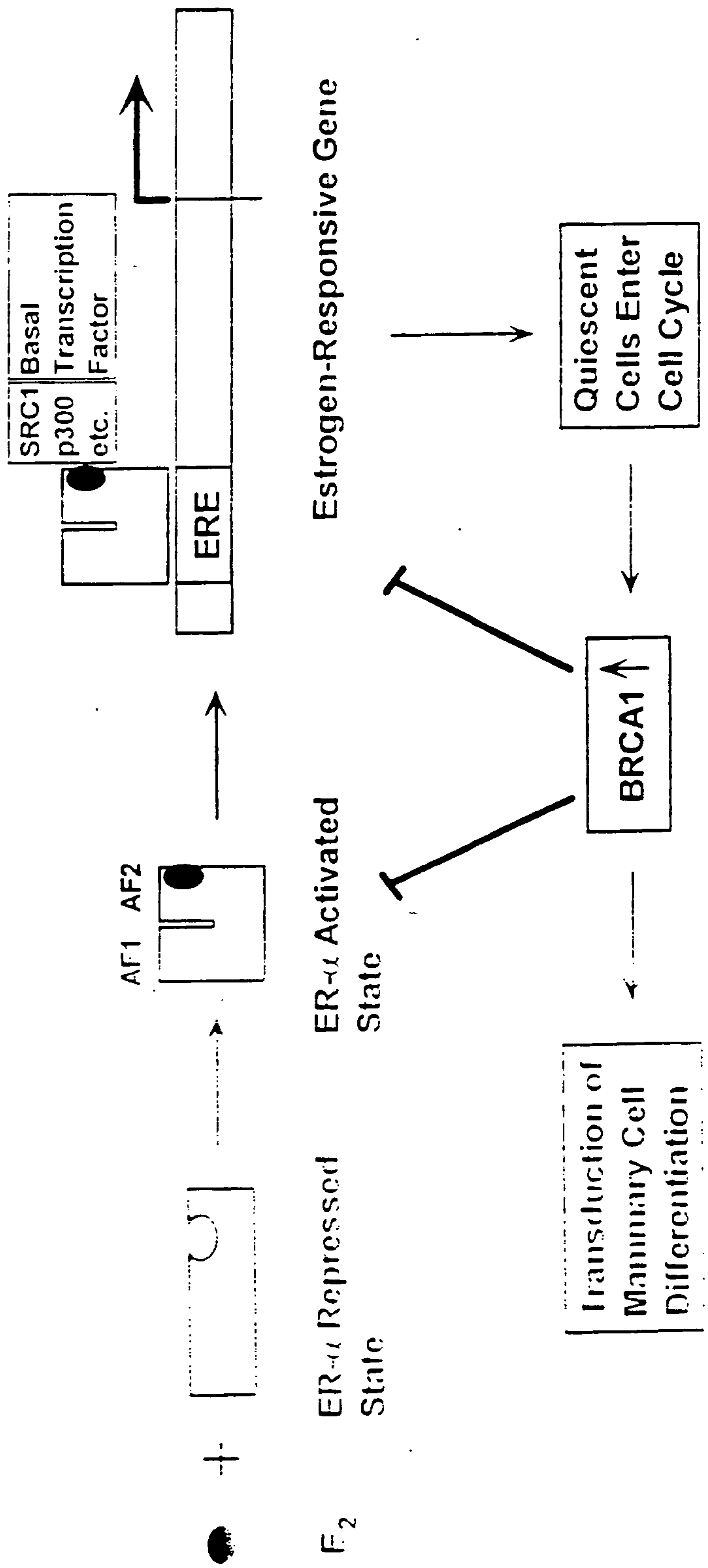

E2
ER-α Repressed State
AF1 AF2
ER-α Activated State
SRC1
p300
etc.
Basal
Transcription
Factor
ERE
Estrogen-Responsive Gene
Quiescent Cells Enter Cell Cycle
BRCA1
Transduction of Mammary Cell Differentiation

FIG.7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/66, 1/68; G01N 33/574
US CL :435/6, 7.23, 8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.23, 8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE CAPLUS BIOSIS EAST WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROMAGNOLO et al. Estrogen Upregulation of BRCA1 Expression with No Effect on Localization. Molecular Carcinogenesis. 1998, Vol. 22, pages 102-109, see entire article.	1-12
Y	PHILLIPS et al. BRCA1 is Expressed Independently of Hormonal Stimulation in the Mouse Ovary. Laboratory Investigation. March 1997, Vol. 76, No. 3, pages 419-425, see entire article.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 JULY 2000	31 JUL 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Cynthea Lawrence For LIN SUN-HOFFMAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196